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CORRECTIONS

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Page 205, line 26; page 207, Table I, last line; page 209, line 20; and page 217, line 12, for *submaxillary* read *sphenopalatine.*

Vol. 68, No. 4, October 1, 1938

Throughout the article by Drs. Pickels and Smadel (pages 583–606), for *photomicrometer* read *microphotometer.*

VASCULARIZATION OF THE CORNEA OF THE RAT IN RIBOFLAVIN DEFICIENCY, WITH A NOTE ON CORNEAL VASCULARIZATION IN VITAMIN A DEFICIENCY

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Plates 1 to 4

Vascularization of the cornea is an early and constant phenomenon in albino rats in riboflavin deficiency. It precedes all other demonstrable lesions of the deficiency.

The vascularization was first observed by us in the routine histological examination of rats deficient in vitamin G (the heat-stable fraction of the vitamin B complex) (1). Subsequent experimentation showed that riboflavin alone was concerned. Control rats subject to the following conditions did not develop corneal vascularization: B_1 deficiency, B_6 deficiency, fasting, old age, maintenance on the experimental diet plus riboflavin.

Cataract has been associated, chiefly by Day and his fellow workers, with vitamin B_2 or G deficiency (the heat-stable vitamin B complex)[1] in rats (2, 3) and in other animals; mice, chicken and monkeys (4). They obtained an incidence of cataract in rats of nearly 100 per cent and regarded this lesion as a better criterion of vitamin G deficiency than dermatitis (3). Recently Day, Darby and Cosgrove (5, 6) showed that cataract in their rats was due solely to riboflavin deficiency.

Cataract was of rare occurrence in our experimental rats. Animals from three different colonies were used as controls against stock variability. The few occasions of its occurrence indicated a litter susceptibility. It also appeared more often in the few animals on which we used grain extracts instead of yeast extract as a source of other essential dietary factors. However, we have made no attempt to duplicate exactly the conditions of the experiments by Day and his associates.

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[1] In this paper, vitamin B_2, vitamin B_2 complex, vitamin G and vitamin G complex as used are all equivalent to the heat-stable vitamin B complex fraction.

1

Other workers also have not been so successful in producing cataract in rats by means of vitamin B_2(G)-deficient diets. Bourne and Pyke (7) obtained only an incidence of 31 per cent. György (8) did not obtain cataract in a large series of rats. Richardson and Hogan (9) rarely saw cataract in their vitamin G complex-deficient rats.

While keratitis is frequently noted in descriptions of vitamin B_2 or G deficiency in rats, there are only meager accounts of the histology of the cornea and only two references to the presence of blood vessels. Pappenheimer describes a section from one of Sherman and Sandel's (10) vitamin G-deficient rats as showing in the cornea "a very slight keratitis, with corneal corpuscles, a few polymorphonuclears, and some new-formed blood channels."

Day, Langston, and O'Brien (2) gave, as their sole histological description of the cornea in vitamin G deficiency, the following: "Microscopic examination of the cornea revealed an inflammatory process in the anterior stroma. The epithelium was normal but small lymphocytic and leucocytic infiltrates accompanied by new blood vessel formation were found directly under the epithelium."

Cataract as a consequence of riboflavin deficiency is probably dependent upon strain susceptibility and certain experimental conditions employed by Day. On the other hand, corneal vascularization invariably occurs when riboflavin is absent from the diet. Corneal changes, observable in gross, indicating the presence of vascularization, have occurred in over 300 rats in a period of more than 2 years. Microscopic examination or India ink injections have never failed to show it after the 4th week of riboflavin deficiency.

Material and Methods

The Rats.—Rats from three different colonies, including Wistar Institute rats, have been used. The employment of three types of breeding rations—(*a*) whole wheat and whole milk powder (Sherman diet 13); (*b*) dog chow and (*c*) scratch feed—has served as a control on the influence of the pre-experimental diet upon the deficiency. In all cases, with the exception of slight time variations, the pathology has been the same. The rats were placed on the experimental riboflavin deficient diet when 21 to 28 days old at 40 to 50 gm. weights.

The Diet.—In our earlier vitamin G-deficient experiments before the multiple nature of the heat-stable vitamin B complex was established (5), the Sherman-Bourquin (10) diet was used. We now know that this diet was primarily deficient in riboflavin (11) but also often contained only small amounts of vitamin B_6 and other essential factors.

The riboflavin-deficient diet which we now employ has the following composition.

	per cent
Casein[2]..	18
Osborne and Mendel salt mixture...................................	4
Cod liver oil...	2
Sugar...	20
Cornstarch[3]..	48
Peanut oil...	8

40 γ of pure vitamin B_1 (thiamin) were given on alternate days.

2 gm. (yeast equivalent) of riboflavin-free yeast extract[4] were given daily, after the first 10 days, as a source of vitamin B_6 and other B factors.

Rats upon this diet cease to grow after 3 weeks but will often live 12 to 15 weeks, occasionally to 18 weeks. The completeness of the diet was proved by the fact that the addition of synthetic riboflavin yielded sustained satisfactory growth without demonstrable pathology in control rats.

The vitamin B_6-deficient diet was the same as the above except that 20 γ of riboflavin was added daily in place of the yeast extract.

Clinical Course

The rats cease to grow after 3 weeks. In 5 to 7 weeks the palpebral fissures become noticeably smaller, the eyeballs are less prominent and appear sunken in the orbits. The lids are slightly swollen and appear to be edematous. The tail becomes dry and scaly. The hair has lost its luster. Soon after there may be small amounts of serous blood-tinged exudate in the conjunctival sac and the lids may become stuck together. The corneas become slightly dull as if finely sanded; after 7 to 10 weeks, one or both corneas may become turbid and white. At about this time, denuded patches begin to appear about the eyes, head, shoulders and back. The skin of the paws and legs becomes dry and scaly. Later, some of the denuded areas become red, moist and even ulcerated, particularly on the paws and upon

[2] Extracted twice by shaking with 60 per cent alcohol and twice by refluxing with 95 per cent alcohol.

[3] Extracted twice by refluxing with 95 per cent alcohol.

[4] 200 gm. of dry brewers' yeast and 1 liter of water were brought, while stirring, to boiling; cooled; 200 cc. methyl alcohol added; supernatant liquid decanted or centrifuged; the alcohol distilled off; HCl added until acid to Congo red; treated twice while stirring with small portions of fullers' earth (tonsil, L. A. Solomon Brothers, New York) until free of riboflavin as tested by absence of fluorescence to ultraviolet light. Distilled at 60°C. or evaporated until 1 cc. equalled 2 gm. yeast.

the head, neck, and shoulders and behind the ears. The distribution and severity of these skin lesions are influenced by scratching and gnawing by the animal. The urine becomes concentrated and highly colored. The animal is dehydrated as indicated by the rapid increase of weight due to water intake when riboflavin is given to a rat still capable of recovery.

The pathology of the skin and other organs of riboflavin-deficient rats will be described in another report. In this we shall describe only the eye in relation to vascularization of the cornea.

The Vascularization of the Cornea

By the end of the 4th week of the riboflavin deficiency, there is a marked radial ingrowth of capillaries into the cornea from the vessels of the limbus. This happens before any change in the cornea visible in the gross has taken place. The transparency is undiminished. While these vessels may be seen by slit-lamp illumination, no turbidity of the cornea or change in the corneal epithelium is revealed by this method or by histological study. By the end of the 10th or 11th week, the blood vessels extend inwards for more than one-third of the diameter of the cornea and some may reach nearly to the center. The time of onset and abundance of the vessels varies, and these differences occurred even among litter mates. Rats which had a pre-experimental diet high in riboflavin developed the corneal vascularization slightly later than those which had a low riboflavin diet (scratch feed). We have followed the initiation, progress and repair of this process by histological sections, injected specimens and by slit-lamp observations, through the cooperation of Dr. T. Gundersen of the Department of Ophthalmology, Harvard Medical School.

Injected specimens gave the most information about the manner of growth of the vessels, their source, and the circulation in the corneal limbus of the normal eye. They were prepared by injecting 25 per cent aqueous India ink, at a pressure of about 75 mm. Hg into the aorta, through the left ventricle of the living anesthetized animal. The whole eye, with the lids and orbital glands, was fixed in 10 per cent formalin, dehydrated in alcohol, and cleared in oil of cedarwood. In some instances, one eye was fixed in Zenker's fluid for comparison by histological study. The cleared injected eye was first studied as a whole. For high magnification and for photographs, the anterior part of the eye was removed by a circumferential cut posterior to the plane of the attachment of the ciliary body. Four radial incisions

were made in order to flatten the curved disc. The iris was next removed. The first preparations made were damaged in the removal of the lids from the eyeball by forcibly tearing away the conjunctiva at its attachment to the globe. This line of attachment extends forward above the limbus of the cornea and when pulled away often carries with it the blood vessels of the limbus. The specimens thus prepared were mounted in compression cells for immediate study. After a few days under pressure they remain flat enough to be used between slide and cover slip.

Source of the Corneal Vessels.—The corneal vessels arise from a rich arterio-venous plexus which encircles the cornea in the limbus and which corresponds to the superficial marginal plexus of the cornea of the human eye.

The arterial supply of this plexus is an encircling artery formed by the anastomosis of the branches of two short trunks which usually arise, one each from the lateral and mesial long posterior ciliary arteries just before these divide to form the greater arterial circle of the iris. Each short arterial trunk divides once into superior and inferior branches which join their fellows of the opposite side. This circular artery of the limbus may have slightly different origins. The main trunks may arise from either branch of the long posterior ciliary artery or each branch of the latter may give rise to an artery. There may be combinations of these methods of origin in the same eye (Fig. 1).

There is great variation in the veins which accompany the circular artery. In general there are two or more roughly parallel freely anastomosing veins, one or more on each side of the artery. In general, the veins lie deep to the artery. The veins empty into several large trunks and communicate as well with the venous plexus of the sclera.

The plexus of the limbus consists of a series of loops, freely inosculating with one another and forming a band 0.2 mm. to 0.3 mm. in width internal to the arterial circle. We have made no attempt at a complete study of this plexus. It is more complex in the young rat than in the old. Fig. 2 is from a normal weight control rat and therefore 3 to 4 weeks old. Fig. 3 is from a normal age control rat and therefore 13 to 14 weeks old. These are representative of a few examples at each age period. The drawings are presented in lieu of an attempt at a more detailed description.

The circular artery of the limbus also gives rise to branches which go to the palpebral conjunctiva, to the nictitating membrane and to the sclera. We could find no branches to the ciliary body and iris.

Manner of Growth of the Corneal Vessels.—The early pattern made by the advancing capillaries, 4th to 7th week of the deficiency, is more complex because of the great abundance of anastomoses (Figs. 4 and 5). The pattern is lace-like and on the advancing border fringed with glomerulus-like loops and arrow-head-like pointed sprouts. Venous connections are even more abundant than arterial and it is impossible to avoid concluding that simultaneous growth from arterial and venous sources takes place. Except for very short pointed sprouts usually situated near the middle of loops, the advancing zone of capillaries is composed

of anastomosing loops. The appearance suggests that the pointed sprouts split behind as they grow, which would be one way of maintaining a pressure gradient. Another possibility is that pool-like expansions of loops advance toward the center of the cornea while contracting on the peripheral side. Lateral bands from arms of loops and from short pointed sprouts give abundant evidence of the establishment of anastomoses by coalescence.

At later periods, 7 weeks or more, (Figs. 6, 7, 8, 9) we find conspicuous radially directed capillaries, arterial and venous in relation to their sources, which are connected by very small calibered vessels, often barely brought out by the India ink mass. This appearance we interpret as due to the closure of many of the early loops. Serial observations on the living animal will be required to determine the exact manner of growth of the capillaries into the cornea. The many publications of the Clarks (12, 13) and their associates and Sandison (14) upon the growth of blood vessels as observed through transparent chambers in the rabbit's ear may well serve as a model for such studies.

The invading capillaries at first lie just beneath the corneal epithelium, but soon others come to lie deep in the tunica propria. By the end of survival time in attempted absolute riboflavin deficiency, 12 to 18 weeks, the blood vessels extend across the cornea nearly to its center.

Behavior of the Blood Vessels in Recovery from the Deficiency

Slit-lamp observation reveals the capillaries in the 4th to 6th week of the deficiency. Turbidity of the cornea does not appear until many days and often several weeks after the vessels are conspicuous under the slit-lamp. Moderate degrees of turbidity of the cornea disappear as soon as 12 hours after giving 60 γ of riboflavin by mouth. Unless severely damaged, the cornea is clear within 48 hours. After 2 weeks' treatment with 20 γ of riboflavin daily, the blood vessels can no longer be seen by an experienced observer with the slit-lamp.[5] They may, however, be demonstrated in sections and by India ink injection. We do not know how long they persist, but they are present in abundance in perfectly clear apparently normal corneas as late as 58 days under adequate riboflavin treatment. They persist long after leucocytic infiltration has disappeared and the tunica propria is restored to normal in all other respects.

[5] 5 γ daily is an adequate protective dose against vascularization.

The earliest recovery period studied by the injection method was 7 days. At this time, evidences of growth activity were almost absent. The glomerulus-like structure, the pools and pointed sprouts, had largely disappeared. The inner border of the vascular zone presented only loops and rare V-shaped loops with pointed extremities. There was marked diminution of caliber of many vessels, particularly of lateral anastomotic branches. A few vessels showed numerous constrictions. In preparations after 12 to 16 days of recovery, the simplification of the vascular pattern was considerable (Fig. 10). All evidences of growth were absent. The constrictions of the radially directed vessels were much more pronounced, producing an effect of beading. By the 25th day of recovery, the pattern was greatly simplified (Fig. 12). The beading effect was generally present. Many long radially directed capillaries now appeared as flattened spirally twisted bands (Fig. 11). The rate of change from the 25th to the 58th day was not so marked and may be summarized as a steadily increasing diminution in number and caliber of the capillaries and a retreat of the vessels from the center of the cornea toward the periphery (Fig. 13).

Correlation of the Injected Specimens with the Histological Changes in the Cornea

1. The Vascularization.—The first capillaries to be seen lie just beneath the corneal epithelium, close to the limbus. Later they appear deep in the tunica propria. They may extend far toward the center of the cornea before leucocytes appear outside of the vessels (Fig. 14). The corneal epithelium remains unchanged until late in the deficiency and then undergoes degenerative changes which we regard as secondary to the lesions in the tunica propria.

Capillaries in cross section have sharply defined circular outlines, and the collagen lamellae are bowed around them. In longitudinal sections, the capillaries are seen to lie between lamellae. The more advanced the deficiency, the more prominent are the endothelial cells and the more numerous are the mitotic figures in the capillary walls. The capillaries can be traced obliquely downward into the depths of the tunica propria; rarely they descend almost perpendicularly to the surface. Only rarely and in very advanced stages of the deficiency have we found the blood vessels deeper than the junction of the middle and lower (deep) third of the tunica propria. While the blood vessels reach a considerable size, they remain capillaries in structure. We could find no indication of the formation of a muscular coat even in the experiments of longest duration, 16 to 18 weeks.

Leucocytes are found in small numbers within a week or two after the vessels have penetrated the cornea. They become progressively more numerous and consist chiefly of polymorphonuclear leucocytes. A few lymphoid cells can be recognized and rarely a mononuclear wandering cell corresponding to the monocyte or mononuclear phagocytic cell. The leucocytes, for the most part, are strung out between the lamellae. They may accumulate in great numbers beneath the

corneal epithelium, especially near the limbus. In advanced stages of the deficiency, great numbers of leucocytes collect in the central non-vascularized portion of the cornea and the epithelium becomes invaded by them (Fig. 15). The collagen of the central portion becomes markedly changed; the collagen fibrils are replaced by a lightly staining non-fibrillary material, as if the fibrils had swollen and fused. When blood vessels have entered such regions, we find an increase of fibroblasts. The newly formed fibroblasts lie adjacent to the blood vessels; there may be numerous ones in sections. There is no special arrangement of these fibroblasts and they may have their long diameter obliquely or perpendicularly aligned with regard to the normal plane of the lamellae of the corneal connective tissue. Rarely we have found areas of densely stained hyaline collagen without fibrillary structure which we have interpreted as evidence of necrosis.

The corneal epithelium over the regions with the most advanced vascularization and infiltration is often markedly changed. The deep layer of cells remains surprisingly persistent. The superficial cells become separated and vesicles may form between the superficial and deep layers of the epithelium. Necrosis and ulceration are very late consequences. The split-lamp, after vascularization is well established but before the cornea becomes turbid, shows at most an occasional point of light reflection, probably indicative of separated superficial cells of the epithelium. Histologically, desquamating cells can be found but not in greater numbers than in the various types of control eyes we have used. Even should this inconstant slit-lamp observation be indicative of an epithelial lesion, it is not conceivable that a lesion of such insignificance and elusiveness should be responsible, in any familiar sense, for the vascularization and subsequent degeneration of the tunica propria.

Throughout the progress of the deficiency, Descemet's membrane and the endothelium covering it show no changes that could be demonstrated by any of a variety of staining methods.

2. Repair.—By the 7th day after restoration of riboflavin to the diet, leucocytes, with rare exceptions, have disappeared from the tunica propria and from the corneal epithelium. The capillaries by this time have become smaller in caliber. After the 12th day of repair, the vessels could not be seen by an experienced observer using the slit-lamp illumination. They can be found with ease in histological sections, though these give no indication of the number and size of the capillaries that are brought to view by the India ink injections.

In general, the study of sections gives the impression that in repair there is a progressive diminution in the number of vessels and in size. It would seem that the pressure used in making the injections opens capillaries that were collapsed. Sections of cornea injected with India ink confirm this impression in that thin lines of the pigment may be found, without accompanying blood corpuscles and with no recognizable endothelial cells, or the latter indistinguishable from the cells of the cornea. Serial sections show that the ink occupies slits of considerable width, indicating compression of the capillaries between the layers of connective tissue lamellae. Careful search is required at all periods of repair to find any evi-

dence of degeneration of capillary endothelium or of entrapped red blood corpuscles.

The former is indicated by a rare swollen finely vacuolated endothelial cell *in situ*. The latter is shown by phagocyted hemosiderin granules. In all corneas, after 2 weeks of repair, all evidences of degenerative changes of the epithelium and of the tunica propria were absent. Fibrin was never found. The appearances in sections, whether of non-injected or of ink-injected eyes, suggest that circulation in part ceases before the endothelium disappears and that the final closure is accompanied or brought about by compression between the connective tissue lamellae. The persistence of vessels for periods up to 58 days, inasmuch as some of these contain normal appearing blood corpuscles and are lined by normal looking endothelial cells, suggests that some of them may persist indefinitely.

Vascularization of the Rat's Cornea in Vitamin A Deficiency

Wolbach and Howe (15) in 1925 described vascularization of the cornea of rats in vitamin A deficiency, accompanied by leucocytic infiltration and changes which were interpreted as edema of the tunica propria. The vascularization was regarded as a phenomenon secondary to the hyperkeratinization of the corneal epithelium and it was suggested that the vascularization was a physiological response to the increased growth rate of the corneal epithelium. We have reviewed the vitamin A deficiency material and apart from the hyperkeratinization of the corneal and conjunctival epitheliums, find great similarities with our findings in riboflavin deficiency. The accumulation of desquamated cornified cells in the conjunctival sac and the consequent inflammatory response might be regarded as adequate cause for ingrowth of blood vessels. However, in vitamin A deficiency, the ingrowth of capillaries takes place concurrently with the epithelial changes. Considerable vascularization may be present before there is any considerable accumulation of desquamated cells and before there are more than early histological signs of inflammatory reaction in the conjunctival limbus. We are disposed to discard an inflammatory explanation of the vascularization. Always, however, the presence of capillaries in the cornea was accompanied by a characteristic change in the corneal epithelium, indicative of the shift to keratinizing metaplasia. The speculation is warranted that an important factor in causation may be a change in permeability of the epithelium in its effect upon the respiration of the cornea as a whole. The details of repair following restoration of vitamin A to the diet

by the addition of butter fat or cod liver oil, though not followe
by the injection method, in general parallel those of repair from
riboflavin deficiency.

The closure of the vessels takes place in the same manner but ap
parently takes place more rapidly. This we infer by the ease wit
which entrapped and disintegrating red blood cells are found. Als
swollen and vacuolated endothelial cells are found with ease and afte
the 2nd week hemosiderin granules are often present within endothelia
cells and strung out in closed capillaries. It has been possible t
follow in serial sections strings of hemosiderin granules into an en
dothelial lined space containing granular deeply eosinophilic re
blood cells, thus confirming the riboflavin repair closure of capillarie
by intermittent constriction. Our impression is that for the period
of repair studied, up to 43 days, obliteration of the vessels due t
vitamin A deficiency is more complete than that due to riboflavi
deficiency.

DISCUSSION

The riboflavin used in the repair experiments and in the purifiec
diets used in control experiments was pure synthetic riboflavin made
by one of us. Therefore, the effects described by us could not have
been due to an impurity.

The clinical observations and histological studies rule out demon-
strable injury of any nature as the inciting factor of the vasculariza-
tion. The rôle of riboflavin as a respiratory carrier suggests that the
vascularization is a response to asphyxia. Since the prevailing opinion
is that the cornea respires through its external surface, the failure
of the epithelium to transport oxygen seems to be an explanation of
the sequence of histologic events. However, it is also reasonable
to assume that the vascularization may be a response to the respiratory
needs of the epithelium itself. If respiration of the cornea is de-
pendent upon the epithelium by virtue of riboflavin activity, the
vascularization in vitamin A deficiency could also be accounted for
on the basis of an altered physiology accompanying the keratinizing
metaplasia. We have not seen vascularization of the cornea of guinea
pigs, either in riboflavin deficiency or in vitamin A deficiency. In
the rat in riboflavin deficiency, there is no vascularization of cartilage.

As a means for the study in a mammal, of growth and regression of capillaries, riboflavin deficiency has obvious advantages which we have made little attempt to explore.   Our only histological observation that may possibly be relevant to the problem is that in common with vitamin A deficiency, there is disappearance of the yellow material in the acini of the Harderian glands.   In both deficiencies, the vascularization is present before the pigment has entirely disappeared.

SUMMARY AND CONCLUSIONS

Vascularization of the cornea of the rat in the absence of antecedent pathology is probably a specific and the most reliable criterion of riboflavin deficiency.

Its initiation and repair may be used for testing the biological activity of compounds structurally related to riboflavin.

The facts that the invading capillaries are easily visible in the living animal and that the growth and regression of the blood vessels are under dietary control and for a considerable period of time unaccompanied by other pathological reactions, make this method very suitable for the study of problems related to capillary growth.

We believe that the best hypothesis in explanation is that the vascularization is a response to asphyxia of the tunica propria.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Drawing of cornea of an injected rat.   77 days on riboflavin-deficient diet.   Reproduced principally to show the circular artery of the limbus.   Figs. 6, 7, 8, and 9 are from this animal.

FIG. 2. Normal rat, weight control.   India ink injection to show the plexus of the corneal limbus.   Arteries black.   Veins stippled.   × about 91.

FIG. 3. Normal rat.   Age control.   India ink injection.   The limbic plexus. Arteries black, veins stippled.   × about 91.

PLATE 1

1

2

3

(Bessey and Wolbach Corneal vascularization in riboflavin lack)
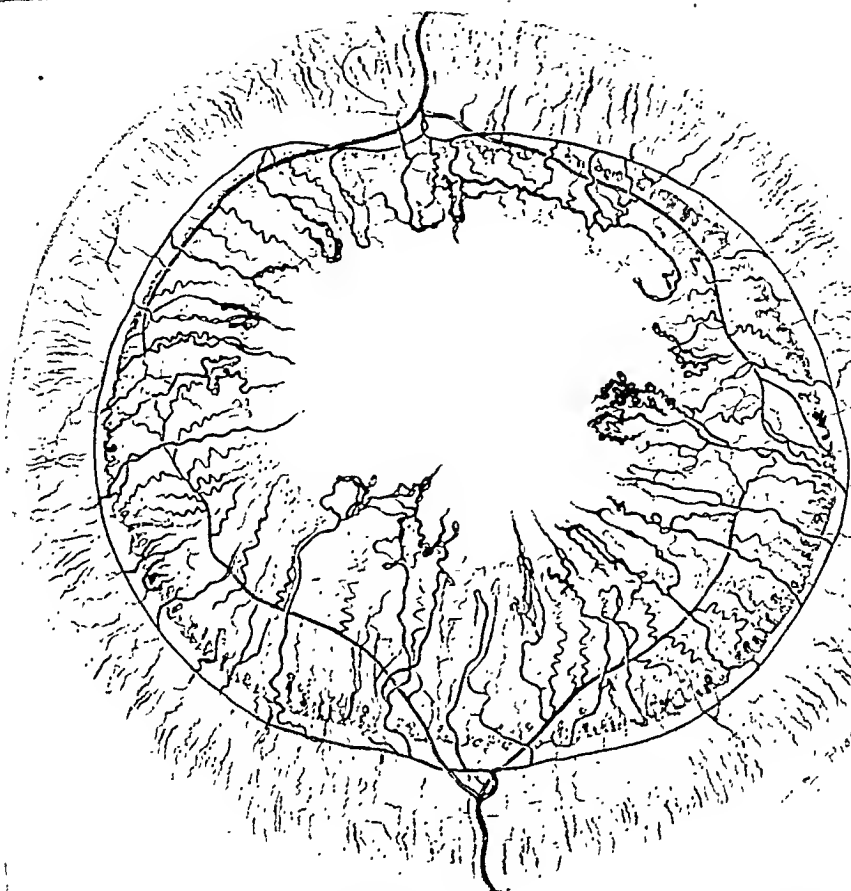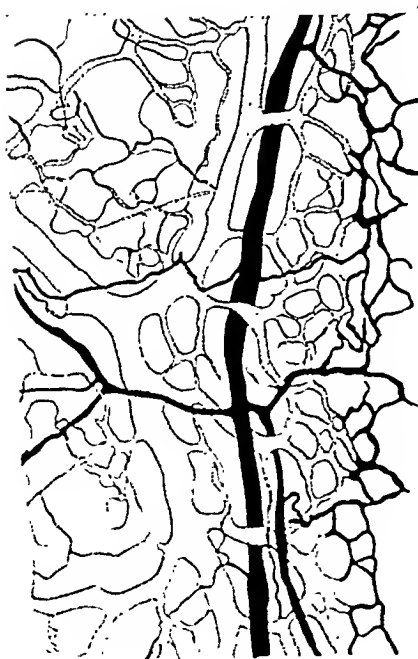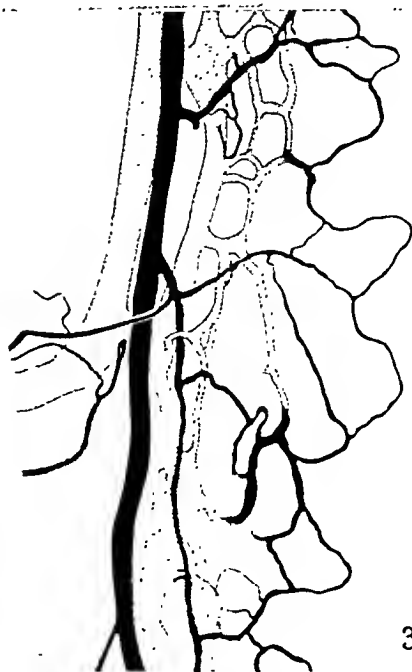
PLATE 2

FIG. 4. Early growth of blood vessels into the cornea. Rat 5 weeks on ribo-flavin-deficient diet. India ink injection. × about 23.

FIG. 5. Riboflavin-deficient rat. 7 weeks. India ink injection. The number of new vessels and the complexity of the pattern are greater than usual. × about 23.

FIG. 6. Same rat illustrated in Fig. 1. Photomicrograph × about 9.

FIG. 7. A detail of Fig. 6. × about 23.

FIG. 8. A detail of Fig. 6. × about 23.

FIG. 9. A detail of Fig. 6. × about 23.
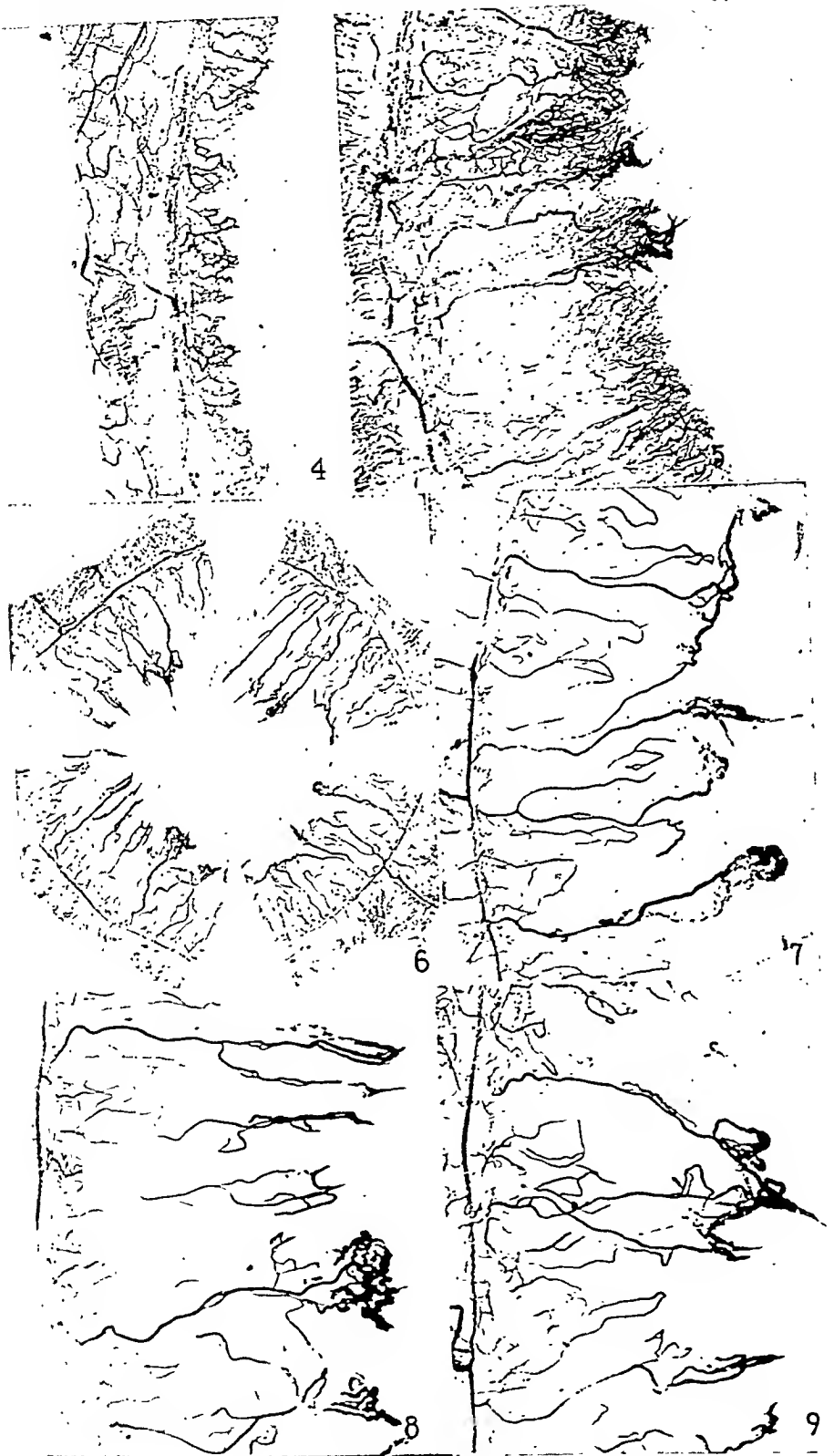
PLATE 2

PLATE 3

FIG. 10. India ink-injected rat.   Corneal vessels showing the circular artery and some of the veins of the plexus.   Riboflavin-deficient rat 91 days, followed by 12 days with riboflavin restored, 40 γ daily.   Shows the cessation of growth and irregular closure of vessels.   × about 32.

FIG. 11. A detail from Fig. 12.   × about 147.

FIG. 12. India ink-injected rat.   Corneal vascularization in repair.   This rat was kept for 56 days on riboflavin-deficient diet and then received 20 γ daily for 25 days.   × about 32.

FIG. 13. Corneal vascularization in repair.   Rat 56 days on riboflavin-deficient diet, then 58 days with the addition of 20 γ daily.   × about 32.
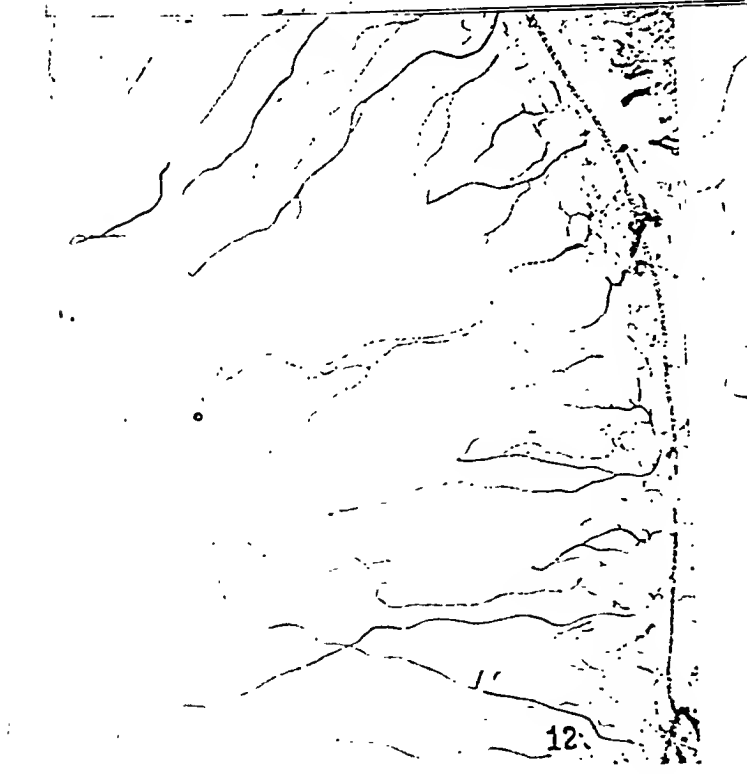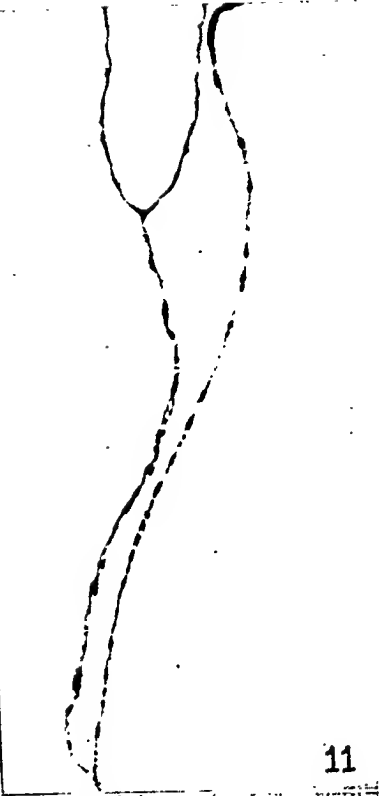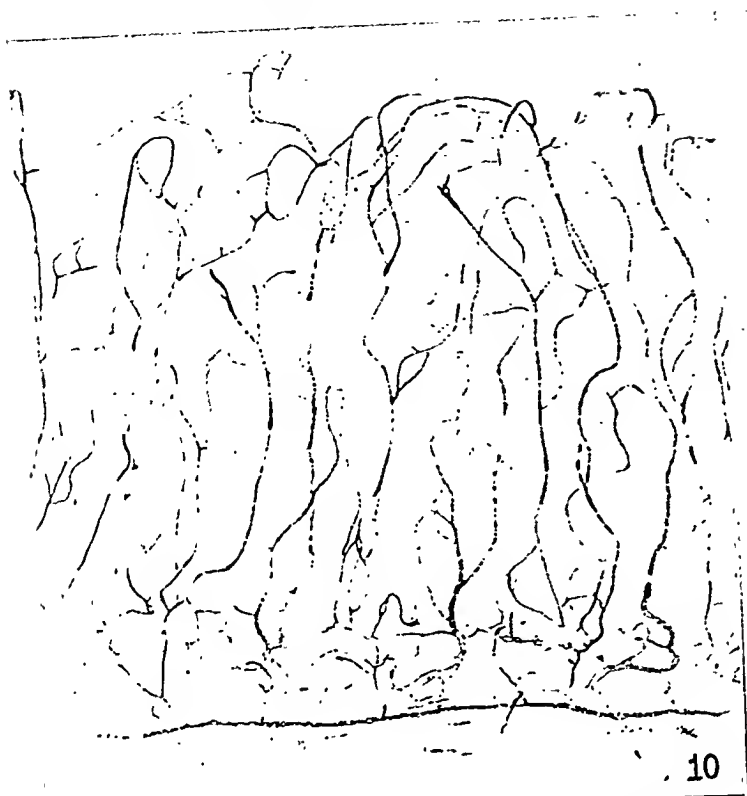
PLATE 4

FIG. 14. Drawing of cornea of riboflavin-deficient rat 70 days. Shows vascularization with beginning leucocytic infiltration. Stained with Mallory's eosin-methylene blue, after Zenker's fixation. × 144.

FIG. 15. Drawings of cornea of rat kept on riboflavin deficient diet with inadequate vitamin B_6 content 120 days. Shows deep vascularization of the tunica propria, heavy leucocytic infiltration and lesions of the epithelium. Stained with Mallory's eosin-methylene blue, after Zenker's fixation. × 144.

PLATE 4

14

15

(Bessey and Wolbach: Corneal vascularization in riboflavin lack)

A NEOPLASM OF MONOCYTES OF MICE AND ITS RELATION TO SIMILAR NEOPLASMS OF MAN*

By J. FURTH, M.D.

WITH THE ASSISTANCE OF C. BREEDIS

(*From the Department of Pathology, Cornell University Medical College, New York*)

PLATES 5 TO 8

A transmissible strain of leukemia in mice with malignant cells related to histiocytes is described in this communication. The strain is derived from a mouse that had granuloma-like infiltrations in the blood-forming tissues. Upon successive passages, the granuloma-like characteristics became less noticeable and the strain was transformed into a neoplasm with malignant cells resembling histiocytes.

Terminology

Experiments described elsewhere (1) led us to conclude that monocytes, histiocytes, macrophages, clasmatocytes, polyblasts, Kupffer cells and microglia cells are synonymous terms for one cell type, which is capable of perpetuating itself by mitotic division. In this communication we shall refer to the round forms of this type of cell seen in the circulating blood, as monocytes, and to all other forms as histiocytes. Tumors of monocytes or histiocytes will be named histiocytomata (monocytoma) and the systemic disease characterized by these cells histiocytomatosis (monocytomatosis). Monocytic leukemia is a synonymous term for leukemic histiocytomatosis (monocytomatosis).

Origin of the Strain

In August, 1937, a 24 months old male mouse (Rfb 385) developed a disease associated with great enlargement of the spleen and slight enlargement of the lymph nodes. The white blood count appeared slightly elevated and the differential count was as follows: Polynuclear and young granulocytes, 60 per cent; small and medium sized lymphocytes, 15 per cent; eosinophiles, 1 per cent. The

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remaining cells, 20 per cent, were mononuclear leukocytes, most of which resembled normal monocytes, but a smaller number, 4 per cent, were unusually large mononuclear cells with a large amount of intensely basophilic cytoplasm and very large nucleus.

The mouse was killed and at autopsy the spleen measured 3.5 by 1.0 cm. in the two greatest diameters; it was firm, gray-red, with numerous nodular gray areas on the external surface and on the cut surface. Most of the lymph nodes were of normal size. The largest nodes were found in the neck and measured 7 mm. in greatest diameter. On microscopic examination there was almost complete replacement of the spleen by large, nodular, partly confluent areas, illustrated in Fig. 3. Most of the cells in these areas resembled normal monocytes with abundant pink staining cytoplasm and an oval or slightly indented nucleus. The size and shape of the nuclei varied greatly but none was hyperchromatic. Occasional giant cells were present. Mitotic figures were few. Scattered among the monocytes was a small number of lymphocytes. The compressed splenic tissue between the nodules formed by the monocytes, was composed mainly of erythroblasts and myeloid cells in various stages of development. The lymph nodes were diffusely infiltrated by mononuclear cells, similar to those in the spleen, and the architecture of the node was distorted. The section of the liver illustrated in Fig. 4 showed extensive nodular and portal infiltration by cells similar to those seen in the spleen. The bone marrow was slightly infiltrated by these cells but here cells resembling fibroblasts were numerous at the sites of infiltration (Fig. 5). A touch preparation of the spleen stained with Wright and Giemsa solution contained mononuclear cells similar to those seen in the section, but the cytoplasm of most of the mononuclear cells was more basophilic than that of normal monocytes.

Transmission Experiments

Inoculations were made with a splenic cell suspension from mouse Rfb 385 into 6 related mice. One of the injected mice died with alterations similar to those observed in the mouse with the spontaneous disease. The disease was passed by intravenous injection from this mouse to 3 of 5 related mice. In successive subpassages most inoculations into related mice were successful.

The results of the first 7 passages are summarized in Table I.

0.1 cc. of a cell suspension containing approximately 10,000 cells per c. mm. was injected into the tail vein of each mouse; it was estimated that each animal received approximately 1,000,000 cells. When the number of cells injected was decreased the number of successful injections likewise decreased and the duration of the illness was lengthened. Most mice injected with 10,000 cells and an occasional mouse injected with 100 cells died of this disease; e.g., in

TABLE I

Transmission Experiments

Passage No.	Material injected	Route	Mice			Length of life after inoculation
			Family	Number injected	Number +	
						days
I	Spleen and lymph node	i.v. and s.c.	Rf	6	1	D 66
II a	" " " "	" " "	Rf	5	3	K 21–57, D 35
II b	" " " "	" " "	x-Rf	6	6	K 36–63, D 28
III a	Spleen	i.v.	x-Rf	5	5	K 23–28, D 14–35
	"	s.c.	x-Rf	2	0	
	"	i.v. and s.c.	Af	8	0	
	"	" " "	x-Af	5	0	
III b	12 day tissue culture	" " "	x-Rf	2	2	K 125, D 125
IV a	Spleen	" " "	x-Rf	2	2	D 16–17
	Frozen and thawed spleen	" " "	x-Rf	3	3	K 32, D 39
	Spleen	" " "	x-Af	10	0	
IV b	"	i.v.	x-Rf	4	4	K 14–25, D 28
	"	"	x-S	2	0	
V	"	"	x-Rf	8	7	K 17–30, D 13–21
VI	"	"	x-Rf	2	2	K 13–76
	"	"	Rf	4	3	K 18, D 18–19
VII	"	i.v. and s.c.	Rf	2	2	D 27–34
	"	" " "	CRf	4	4	K 25, D 34–39
VIII	"	i.v.	CRf	3	2	K 15–27
IX	"	"	Rf	5	5	K 15, 15, D 20–23
	"	"	RfAk	4	4	D 16–28
	"	"	Ak	8	0	
X	Cell-free supernatant	"	x-CRf	11	0	
	Sediment	"	x-CRf	5	5	D 8–13

Abbreviations Used in the Tables.—K = killed; D = died; i.v. = intravenous; s.c. = subcutaneous. The origin of the stocks of mice named Rf, Af and S has been described (2). Stocks Ak, Af, S and C are unrelated to stock Rf in which the spontaneous disease originated. CRf is a first generation hybrid between the C stock of the Roscoe Jackson Memorial Laboratory and our Rf mice, and AkRf are similar hybrids of the corresponding stocks. x means that the mice were x-rayed from 1 to 7 days before inoculation with approximately 400 r.

one experiment the 2 mice that received 1,000,000 cells died 17 days after injection, 2 of 3 mice receiving 10,000 cells died after 27 days, and 1 of 4 mice receiving 100 cells died after 29 days.

None of the 23 unrelated mice (Table I) that were injected developed the disease even though 17 of them were irradiated with 400 r before inoculation. Mice of the first generation of hybrids between resistant and susceptible stocks were susceptible.

The material introduced into the subcutaneous tissue produced either a small, nodular growth at the site of injection or no lesion detectable in the gross. In this respect this strain resembled the transmissible chloroleukemia described by Hall and Knocke (3).

All 3 mice of the related stock Rf injected with splenic tissue that had been frozen slowly and kept during 1 hour at −70°C. died with monocytoma. This result may be explained by the resistance of these cells to slow freezing (4).

Attempts to Transmit the Neoplasm with Material Free from Living Cells

1. Splenic tissue dried *in vacuo* in the frozen state failed to produce the disease in 4 irradiated mice of stock Rf. 3 months later the · same mice received additional irradiation and were inoculated intravenously with live malignant mononuclear cells. Monocytoma characteristic of this strain developed in all 4 mice from 11 to 40 days after the reinjection.

2. 10 irradiated mice received intravenous injections of unfiltered cell-free splenic extract, obtained by spinning a thick splenic suspension at 3500 R.P.M. and 4 control mice received the sediment. The control mice died with monocytoma from 8 to 13 days after injection; the mice that received the cell-free extracts remained healthy.

3. Previous experiments have suggested that irradiation may be used to discriminate cells from virus in inocula containing both (5). Viruses are resistant to x-rays, whereas cells of mice present in the material are destroyed by approximately 500 to 6000 r of x-rays. X-rays do not bring about immediate destruction of the cells, but they may survive for several days and even multiply, so that viruses, should they be present within the cells, have an excellent opportunity to obtain a foothold in the new host.

In one experiment 4 mice received, with no ill effect, an intravenous injection of approximately 1,000,000 cells, exposed to 2000 r, and 4 mice a similar number of cells exposed to 4000 r. 2 control mice that received the same number of unirradiated cells died with this disease 17 days after injection; 2 of the mice that

had received approximately 10,000 cells each died after 25 and 28 days respectively; and one of 4 mice receiving approximately 100 unirradiated cells died after 29 days. In a second experiment that will not be described in detail, approximately 1,000,000 cells that were exposed to 1000 r and 2000 r respectively were injected into each of 4 mice, with no ill effect. The control injections were as effective as in the first experiment.

These experiments do not support the opinion that there is a virus in this material capable of producing monocytoma under the circumstances of our tests.

Anatomical Changes

The gross characteristics of the disease are illustrated in Figs. 1 and 2. In the advanced stage, the liver was enormously enlarged, greatly distending the abdomen. It was thickly spotted with minute round or irregular gray-white, red and yellowish gray areas. The gray areas were due to the presence of minute masses of the malignant mononuclear cells, the red areas to hemorrhage, and the yellowish gray opaque spots to necrosis. Usually all of these alterations were present, although occasionally some were inconspicuous. The spleen was moderately or greatly enlarged and was the site of similar alterations. A few lymph nodes, usually the cervical, were slightly or moderately enlarged; others were normal in size. The lungs showed small or extensive spotty areas of hemorrhage and occasionally yellowish gray tumor nodules.

The microscopic findings in the mouse with the spontaneous disease, and in the first few subpassages, have already been mentioned. In the spontaneous disease and in mice of the second subpassage, the microscopic examination of the blood-forming organs showed collections of mononuclear cells, resembling those seen in infectious granulomata of man. Most of these monocytes were not hyperchromatic (Fig. 6). The cytoplasm was abundant, pink staining, and the shape of the nucleus varied from vesicular to multilobed forms. In later passages these cells were few, whereas cells with larger hyperchromatic nuclei and relatively less but more basophilic cytoplasm were present in larger numbers. The shape of the nucleus varied greatly from vesicular to multilobed forms, shown in Figs. 12, 13 and 17. The photographs do not faithfully reproduce the lobations of the nucleus

because the cells were so large that the nuclear shape could be ascertained only by focusing at different levels on the same cell.

The infiltration in the liver was diffuse, usually with the formation of nodular tumor masses. The malignant cells often invaded vessels of medium size, almost completely occluding them. Mitotic figures were present in large numbers. Cells other than variants of the malignant monocytes were few in the tumor-like infiltrations. In the spleen the infiltration was equally extensive and almost completely replaced the pulp. A small amount of lymphoid tissue and of granuloblastic and erythrogenic foci often persisted about the follicles and trabeculae. In mice that died at an early stage of the disease the infiltration was limited to the splenic pulp. The infiltration in the lymph nodes was scant or often absent. It formed nodular growths that first appeared about the lymph sinuses. The degree of involvement of the bone marrow was variable, being usually scant. In an occasional instance the greater part of the femoral marrow in the sections examined was replaced by neoplastic cells. In the first few subpassages a tendency to stimulate connective tissue growth was noted; this was less conspicuous in the course of the later passages. When introduced into the subcutaneous tissue the malignant cells either produced no gross alterations or a small tumor measuring from 2 to 5 mm. in the greatest diameter, and the mice died with extensive metastases in the blood-forming tissues, similar in appearance to those found in mice that had been injected intravenously. Microscopic examination of these subcutaneous growths showed that there was invasion of the skin and subcutaneous tissue by malignant cells, with extensive areas of hemorrhage and necrosis.

In 2 mice a chronic disease was produced by the introduction of a tissue culture 12 days old into the subcutaneous tissue. When killed 125 days after the injection one mouse had a small nodular growth at the site of injection, measuring approximately 8 mm. in greatest diameter and there was no evidence of infiltration in the internal organs. The second mouse that died 125 days after the injection had, in addition to a similar local growth, a generalized neoplasm with tumor nodes composed of monocytes, many of which resembled the less hyperchromatic type of cell seen in the spontaneous disease and in the earlier passages.   .

The malignant cell evidently may assume many different forms (Figs. 3, 6, 7, 12, 13, 17). Giant cells like those seen in Hodgkin's

disease were found in large numbers in occasional mice (Fig. 13).
The factors that determine the morphological characteristics of the
malignant cells of the strain require further study.

Occasionally in mice in which the disease had a relatively long con-
tinued course there was slight or moderate fibrosis in the tumor-like
infiltrations. In sections of these lesions stained with Foot's modifi-
cation of Masson's trichrome stain there was a moderate amount of
green staining amorphous material with occasional fibrils between the
neoplastic cells. In sections stained with Foot's stain for reticulum
a moderate number of reticular fibers were seen between the neo-
plastic cells.

Alterations in the Blood Picture

In spite of advanced invasion of medium sized vessels of the liver
by malignant histiocytes, these cells were present in the circulating
blood in only small numbers. In the blood smear of 10 mice that
were x-rayed before inoculation the percentage of presumably malig-
nant monocytes varied at the height of the disease from 0 to 37 with
an average of 10.4 per cent and in 10 not x-rayed mice from 0 to 23
with an average of 3.5 per cent. The malignant monocytes differed
from normal monocytes mainly by the large size of the nucleus and
basophilia of the cytoplasm. Cytoplasmic granules were not seen,
with the exception of occasional fine azurophile granules, and the
cells did not give the oxydase reaction. Similar cells are usually
designated as monoblasts. In fixed preparations many of these
large mononuclear cells became smudged but were still recognizable
by the outline of the large nucleus and by intensely basophilic cyto-
plasm. Touch preparations of infiltrated organs contained these
cells in large numbers. The total leukocyte count was only slightly
elevated. An occasional conspicuous rise of the leukocyte count was
due to associated leukocytosis. Table II gives examples of the blood
counts during the course of this disease. The largest number of
malignant cells was found in mouse CRf 65.

Characteristics of the Malignant Cells

The behavior of histiocytes in tissue cultures (1) is highly char-
acteristic. Whether derived from the brain (microglia cells) or liver

(Kupffer cells) or from the blood (monocytes), these cells migrate into the explant and assume at first the form of the resting microglia cell of the brain; they become transformed into epithelioid cells or fat-laden round cells (the compound granular corpuscle), may form

TABLE II

Blood Counts of Mice With Histiocytomatosis and of Control Mice

Mouse No.	Date of			Red cell count in millions	White cell count in thousands	Differential count					
	Irradiation	Injection	Examination			Lymphocytes	Polynuclears	Monocytes	Malignant cells		Unclassified
									Preserved	Smudged	
CRf 58 (killed 5/26)	5/11	5/11	5/13	12.9	4.7	59	39	1	0	0	1
			5/20	10.5	9.1	37	60	1	0	2	0
			5/26	8.6	11.4	39	32	8	15	3	3
CRf 59 (killed 6/7)	5/11	5/11	5/16	9.6	2.0	53	40	5	0	0	2
			5/24	12.6	9.1	22	66	6	0	0	6
			5/31	9.0	38.8	11	75	8	3	0	3
			6/7	7.0	13.1	12	46	5	10	15	12
CRf 65 (killed 6/1)	5/11	5/11	6/1	7.4	76.2	20	30	4	25	12	9
CRf 62	5/11	—	5/16	7.4	2.5	58	33	4	0	0	5
			5/24	7.8	3.1	78	20	2	0	0	0
			6/7	8.2	4.1	20	74	4	0	0	2
Rf 706 (died 6/11)	5/26	—	5/26	8.8	16.0	44	50	6	0	0	0
			6/2	10.0	13.0	59	35	6	0	0	0
			6/9	6.9	51.8	30	57	10	0	0	3
CRf 34 and 35	—	—	*	8.4	8.2	55	42	2	0	0	2

* Average of 6 counts made at from 3 to 7 day intervals on these 2 mice.

multinucleated giant cells of the foreign body type, and may be actively phagocytic. They are not transformed into any other type of blood cell or into fibroblasts.

The malignant mononuclear cell of a transmissible neoplastic

disease of mice previously described (6) had many of the character-
istics of monocytes and formed in tissue cultures peculiar multinuclear
giant cells. *In vitro* the malignant monocytes described in the present
communication, behaved in a characteristic manner. Unlike fibro-
blasts and endothelial cells they did not anastomose, but like blood
cells migrated singly from the explant. The cytoplasm became
elongated with bulky bulbous projections. The microglia-like forms
with slender, long drawn out and branching processes usually seen
in cultures of normal monocytes were not observed. The shape
of the nuclei did not deviate conspicuously from that seen in sections
of the blood-forming organs of animals with this neoplasm (Figs.
8–11). In an occasional culture the cells were somewhat spindle-
shaped but did not form a reticular network. The large number of
mitotic figures, as many as six in a high power field, seen occasionally
in cultures of the buffy coat, was evidence that these cells proliferated
by mitotic division. The morphologic appearance of these cells
in tissue cultures was less variable than that of normal monocytes.

Lewis (7) compared the malignant monocytes of strain Rfb 385
with those of strain C 57 which she has recently isolated. She ob-
served that the characteristic cell of strain Rfb 385 behaved more like
a large macrophage that had become malignant, while that of mono-
cytoma C 57 was more like an epithelioid cell that had taken on can-
cerous properties. Besides the specific cell, both lesions contained
some cells of the monocyte, macrophage, and epithelioid cell type.

Phagocytosis.—One of the significant characteristics of the various
forms of monocytes is their power of phagocytosis. Phagocytosis
by the malignant cells of this strain was studied both *in vivo* and
in vitro.

Normal and leukemic mice were given repeated intravenous injec-
tions of India ink and the animals were killed a few hours after the
first injection. The Kupffer cells in the liver of the normal mice
were studded with carbon particles. In the normal spleen the pres-
ence of a perifollicular zone formed by phagocytes was noted in all
sections. Phagocytic mononuclear cells were less numerous in the
pulp than in this perifollicular zone. Phagocytes were scant in the
foci of myeloid metaplasia present in many normal spleens.

S 2 and tend to form giant cells with lobed nuclei.   The numerous mitotic figures among these cells and their invasion of fatty tissue in absence of other cells strongly support the view that monocytic leukemia of man is the result of unrestricted multiplication of monocytes.   No. 680 of this collection diagnosed by two competent pathologists as reticular cell sarcoma and by another as Hodgkin's sarcoma, is an example of histiocytic neoplasm with no blood involvement.   This neoplasm is characterized by monocyte-like cells whose nuclei are often indented or lobed or convoluted; these cells form multinuclear giant cells resembling those of strain S 2.   No. 637 of the Lymphatic Registry is characterized by similar cells.

Downey (12) has described several cases of monocytic leukemia and his colored figures (Nos. 17 to 25) illustrating these malignant human monocytes resemble the malignant monocytes of strain Rfb 385 so closely that these cells from men and mice can barely be distinguished.

Autopsies have been performed in recent years in this department on two human cases of this type of neoplasm and Figs. 14 to 16 illustrate the malignant cells in them.

Neoplastic cells like those shown in Fig. 16 infiltrated the skin of a woman 36 years old causing swelling and redness in focal areas.   The blood picture was not significantly altered.   The spleen and lymph nodes were not enlarged.   The postmortem diagnosis[2] was reticulo-endothelial (retothelial) sarcoma with involvement of skin, lymph nodes, tonsils (Fig. 16), spleen (weight, 680 gm.), bone marrow, mesenteric, omental and retroperitoneal tissues, pleurae, trachea, bronchi and gastro-intestinal tract.   The cells infiltrating these structures had a large vesicular and slightly indented or lobed hyperchromatic nucleus.   A nucleolus was present in only a few cells.   The cytoplasm was moderately abundant and the relation of nucleus and cytoplasm varied greatly.   Occasional cells had two nuclei.   Eosinophiles were present in small numbers and slight fibrosis occurred at the site of occasional infiltrations.   In a section stained with silver nitrate there was a variable and usually moderate amount of reticulum among the tumor cells. The observation that there is in a few places little if any reticulum between large groups of cells suggests the possibility that much of the reticulum is derived from preexisting reticulum.

The cells in Figs. 14 and 15 illustrate the malignant cells in a neoplasm of a man 37 years old[3] diagnosed as round cell sarcoma with involvement of the spleen, of the stomach with extensive necrosis and ulceration, of the intestinal tract with hemorrhage into the lumen.   The malignant cells in this case (Figs. 14 and 15) resemble the neoplastic histiocytes of mice of strain Rfb 385 (Fig. 17).   Both are large round cells with large hyperchromatic vesicular and indented or lobed nuclei and a moderate amount of slightly basophilic cytoplasm.   The disease in both instances is characterized by an unrestricted growth of these cells.

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[2] Made by Dr. Lawrence Smith.

[3] Autopsy performed by Dr. J. W. Hall.

DISCUSSION

Relation of Histiocytes to Other Cells.—It is beyond the scope of this work to review the different opinions on this highly controversial subject; this has been done recently by Forkner (10), Jaffé (11), Downey (12), Hadfield and Garrod (13) and others. It seems desirable, however, to summarize the author's opinion based on a critical study of the literature and on experiments of his own.

The existence in the adult organism of an ancestral cell of the mesenchyme which gives rise to the well recognized cells of the bone marrow and blood is now almost universally accepted. These cells are believed to form the framework of the blood-forming organs, and according to Maximow, the main exponent of this view, produce monocytes. This supposition, based on observations of fixed and stained preparations, has to be reconsidered in the light of recent studies of cells in the living state. Clark and Clark (8 a) observed that histiocytes develop in the tissue of the tail of transparent axolotl larvae from an undifferentiated strand of primitive mesoderm ventral to the notochord at the same time as do other varieties of early connective tissue cells. They have observed furthermore the increase of histiocytes by mitotic division but have not seen them change into any other form of blood or tissue cell. The source of monocytes in later life, they suggest, must be looked for in some specialized region.

The opinion that monocytes arise from lymphocytes has been discussed elsewhere (1 b), and in accordance with the observations of Clark and Clark and Lewis and Lewis the conclusion has been reached that lymphocytes do not form monocytes.

Although there is ample evidence indicating that histiocytes are derived from histiocytes and pure cultures of them have been grown *in vitro* by numerous workers, their derivation from other cells during postembryonal life and their ability to form fibroblasts is doubtful.

In sections of mice that had been injected with India ink the phagocytic cells containing carbon particles are scattered about the lymphoid follicles and in the pulp and appear to line the blood sinuses of the liver and spleen; but it is questionable if histiocytes are identical with the lining cells of these blood sinuses.

Our studies made in association with Dunning and those of other investigators quoted indicate that Kupffer cells are not identical with the endothelium of the sinusoids of the liver. The studies of Eliot (14) are of unusual interest in this connection. She injected into the veins of rabbits monocytes that had phagocytosed carmine particles and found that these cells became established about the sinusoids of the liver and were indistinguishable from normal Kupffer cells. These studies have been confirmed by de Haan and Hoekstra (15). The experiments of Rous and Beard (16), on the other hand, indicate that monocytes, histiocytes and Kupffer cells, although genetically related, differ morphologically to no slight

extent as also in physical properties, *e.g.* stickiness, and in ability to survive outside the body.

MacCallum (*cf.* 8 *b*) and several other workers found that endothelium possesses phagocytic ability and Clark and Clark (8 *b*) observed in the transparent tails of tadpoles phagocytosis of carmine and carbon particles by connective tissue and endothelial cells. Direct observations made upon living tissue by Clark and Clark (8 *c*) indicate that endothelial cells do not form monocytes and the contradictory reports of several workers, reviewed by Clark and Clark, are not convincing.

In sections a sharp differentiation of endothelial cells from phagocytic mononuclear cells such as Kupffer cells is not possible. In tissue cultures, on the other hand, the two types of cells, histiocytes and fibroblast-like (endothelial cells and fibroblasts), can usually be distinguished. Differentiation of endothelial cells and fibroblasts in tissue cultures, we believe, is uncertain. We have noted in the cultures of the buffy coat of the blood of animals that received intravenous injections of India ink all forms of phagocytic cells that were present in the cultures of the spleen of the same animals. Phagocytosis by fibroblast-like cells was inconspicuous. Histiocytes and fibroblast-like cells have been grown separately in pure cultures by several investigators; a few of them have stated on insufficient evidence that there is occasionally transformation of one cell type into the other (*cf.* 17). Direct observation of cells in the transparent chamber of the rabbit's ear by Clark and his associates and numerous studies on the behavior of histiocytes in tissue cultures indicate that endothelial cells are not related to histiocytes and suggest that histiocytes are not able to form a sinusoid or an anastomosing network of tissue. It seems more likely that fibroblasts and endothelial cells form the permanent framework of the blood-forming organs and about these cells are large numbers of motile histiocytes. Both lymphocytes and monocytes seem to be independent self-perpetuating strains of cells (*cf.* Lewis, 8 *b*).

Neoplasms of Histiocytes.—If monocytes are capable of perpetuating themselves by mitotic division it is possible that they can undergo malignant transformation and undoubted cases of such neoplasms are on record (*cf.* 12). These may occur in the form of solitary tumors (histiocytoma) or as a systemic disease (histiocytomatosis) involving mainly the blood-forming organs with or with no leukemia. Most human neoplasms of histiocytes have been described under the term reticulum cell sarcoma or reticulosis, leukemic and aleukemic (*cf.* Krumbhaar, 18) but since the relation of histiocytes to reticulum fibers and to the reticular fibroblast-like cells of the blood-forming organs is obscure this terminology is not desirable.

The origin of the reticulum of sarcoma has not been definitely established (*cf.* Arey, 19). The reticulum of sarcoma may be remnant of preexisting reticulum

or it may be newly formed by either the stroma cells or the neoplastic cells themselves. Schwann, Flemming and others derive reticulum from the ectoplasm of cells; Henle, Koelliker and others from a semifluid amorphous substance secreted by the cell. Some more recent workers assume that an enzyme of the fibroblasts passing into the surrounding fluid jelly is responsible for fibril formation but others attribute it to a coagulation process in the intercellular substance and ascribe no specific rôle to the fibroblasts (*cf.* Arey, 19). There is not enough evidence that quantity or appearance of reticulum characterizes a distinct type of neoplasm that could be named reticulum cell sarcoma. Our studies do not support the opinion that the reticular fibroblast-like cells of lymphoid tumors are neoplastic elements of the growth (20). They indicate that lymphoid, myeloid and monocytic leukemias are the result of unrestricted proliferation of lymphocytes, immature myeloid cells and monocytes, respectively.

For the reasons given it seems desirable to abandon the term reticulum cell sarcoma and name the solitary growth of histiocytes histiocytoma or monocytoma, and the systemic disease histiocytomatosis or monocytomatosis. Either may be leukemic or aleukemic. The transmissible neoplasm here described is sometimes aleukemic and sometimes leukemic but with small numbers of malignant cells in the peripheral blood. Monocytic leukemia may be substituted for the cumbersome term leukemic histiocytomatosis.

The Relation of This Transmissible Histiocytoma of Mice to Hodgkin's Disease.—The available evidence is not sufficient to prove that Hodgkin's disease is neoplastic in nature although its characteristics strongly suggest this view. A disease exactly like it has not been found in animals, and the experience gained in the study of transmissible neoplasms of animals including those produced by viruses does not elucidate the morphological characteristics of Hodgkin's disease.

Nevertheless, there are certain similarities between Hodgkin's disease and the neoplasm of mice described here.

Both may have granuloma-like characters though they are readily distinguishable. In the mouse with the spontaneous disease and in a few instances of the transmitted disease there was in the blood-forming organs a focal and diffuse overgrowth by large mononuclear cells like histiocytes among and about which were lymphoid cells in variable numbers. Among the mononuclear cells were occasional giant cells; plasma cells and eosinophiles were seen in a few places. There were areas of necrosis and an increase of fibrous connective tissue in some of the foci of infiltrations. In the course of successive passages the pleomorphic characteristics of these alterations disappeared almost completely and the trans-

missible strain changed into a disease unquestionably neoplastic. It is well known that the lesions in Hodgkin's disease are sometimes pluricellular and granuloma-like, sometimes monocellular characterized by an overgrowth of large mononuclear cells with giant cells named after Sternberg and Reed. Similar giant cells occur in the mouse neoplasm.

Occasionally a tumor definable by one pathologist as a histiocytoma has been designated Hodgkin's disease by another, *e.g.* No. 680 of the Lymphatic Registry already cited. Moreover, Doan and Weisman (21) have pointed out that cases that are clinically monocytic leukemia may possess the anatomical characteristics of Hodgkin's disease.

The mononuclear cells of Hodgkin's disease are not known to be phagocytic. Phagocytic ability of the malignant mononuclear cells of this strain Rfb 385 could be demonstrated only by special tests. It is noteworthy that India ink injected intravenously into mice with this neoplasm was taken up almost exclusively by normal histiocytes.

The mononuclear cells of Hodgkin's disease do not appear in the blood but the malignant mononuclear cells of this neoplastic disease of mice are in many instances present in the blood in small numbers during the terminal stage of the disease.

The transmissible disease here described is characterized by proliferation of mononuclear cells which resemble the monocytes of the blood. Proliferation of these malignant monocytes is occasionally associated with stimulation of other cells when the disease is granuloma-like and resembles Hodgkin's disease but is not identical with it; more often the growth of these cells is unaccompanied by those of other cells, the malignant cells appear in the blood in small numbers, and the disease closely resembles monocytic leukemia.

SUMMARY AND CONCLUSIONS

A transmissible neoplasm of mice characterized by malignant cells resembling histiocytes (monocytes) is described. The morphology of these cells and the microscopic appearance of the lesions are similar to those of human neoplasms formed by histiocytes.

The malignant histiocytes form tumor-like masses in the liver and spleen and infiltrate these and other tissues. They are present in small numbers in the blood of many mice when the disease is far advanced. The malignant cells have scant phagocytic ability. The fixed cells of the host (endothelial cells and fibroblasts) have no significant part in the production of the lesions of the disease.

Transmission is readily accomplished when material containing

the malignant histiocytes is used for inoculations, but fails in their absence. Attempts to demonstrate a cell-free transmitting agent have been unsuccessful.

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EXPLANATION OF PLATES

The sections were stained with hematoxylin and eosin. The blood smears were stained with Wright and Giemsa solution. The magnifications given are approximate.

PLATE 5

FIG. 1. Transmitted monocytomatosis with greatly enlarged liver and spleen. These organs are spotted with areas of hemorrhage.

FIG. 2. Transmitted monocytomatosis with numerous partly confluent grayish tumor nodules in the liver.

FIG. 3. Granuloma-like infiltrations in the spleen compressing the pulp in the mouse with spontaneous monocytomatosis (Rfb 385). × 250.
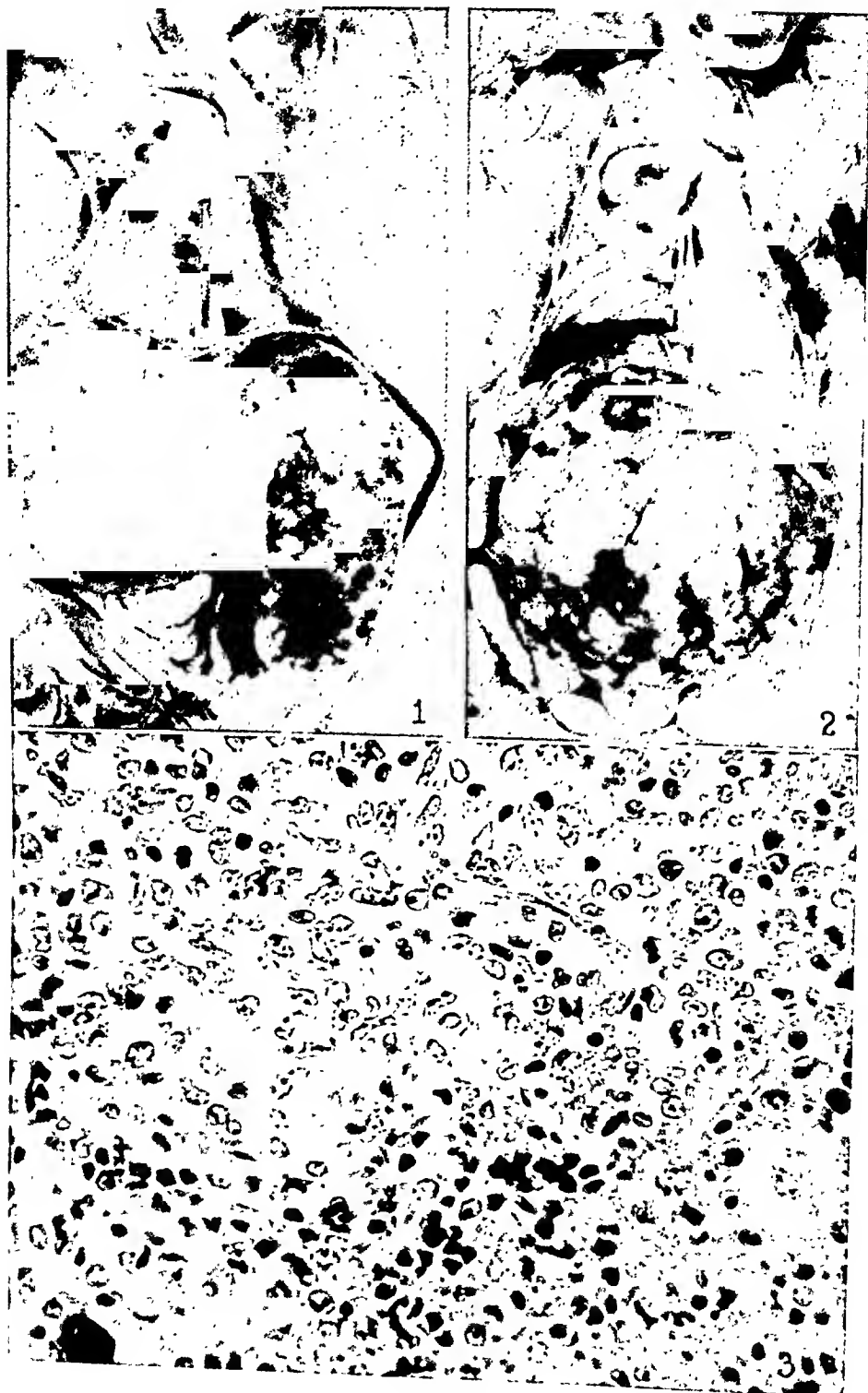
PLATE 6

FIG. 4. Portal infiltration in the liver of a mouse with the spontaneous mono-cytomatosis (Rfb 385); the cells resemble those that infiltrate the spleen.   × 75.

FIG. 5. Focal infiltration of the bone marrow with mononuclear cells and early fibrosis in the same mouse.   × 150.

FIG. 6. Infiltration by large mononuclear cells with abundant, slightly hyper-chromatic cytoplasm in the lymph node of a mouse of the second passage.   × 350.

FIG. 7. Infiltration similar to that shown by Fig. 6, but with more hyper-chromatic mononuclear cells, some of which are phagocytic, in a lymph node of a mouse of the third passage.   × 400.
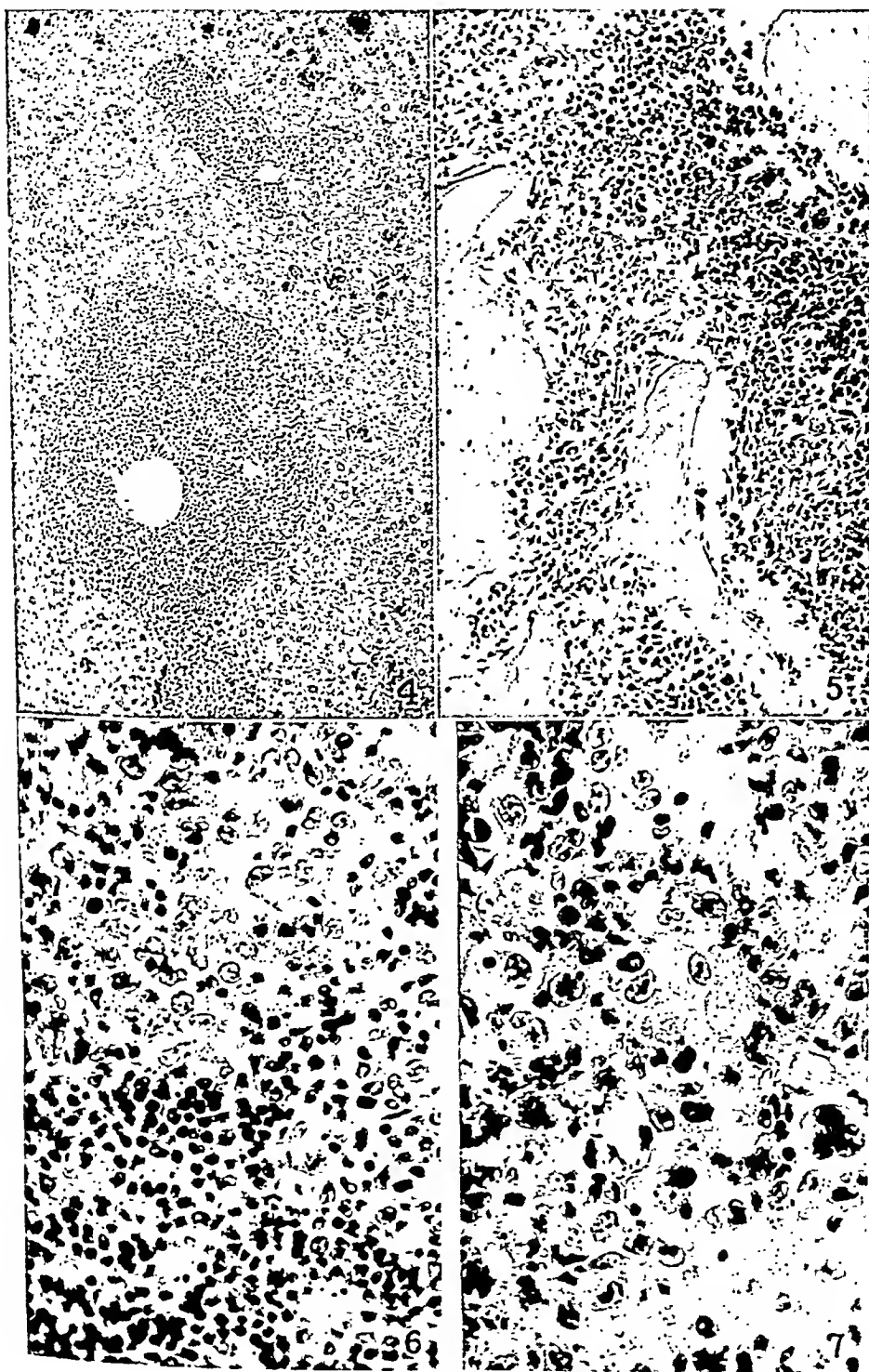
FIG. 8. 3 days old tissue culture of the buffy coat of blood of a mouse with monocytomatosis, that received intravenous injections of India ink. The black granules in the cytoplasm of the cells are phagocytosed particles of India ink. × 400.

FIG. 9. Touch preparation of the liver showing numerous malignant mononuclear cells. × 1000.

FIGS. 10 and 11. Malignant mononuclear cells in a blood smear of a mouse that received intravenous injection of India ink. The black granules in the cytoplasm are those of India ink. × 1000.

FIG. 12. High magnification of the malignant cells in a tumor-like infiltration in the liver (fifth passage). There is a giant cell in the field. × 800.

FIG. 13. Microscopic section of a tumor-like nodule in the liver of a mouse with transmitted leukemia (third passage), showing numerous giant cells. × 400.
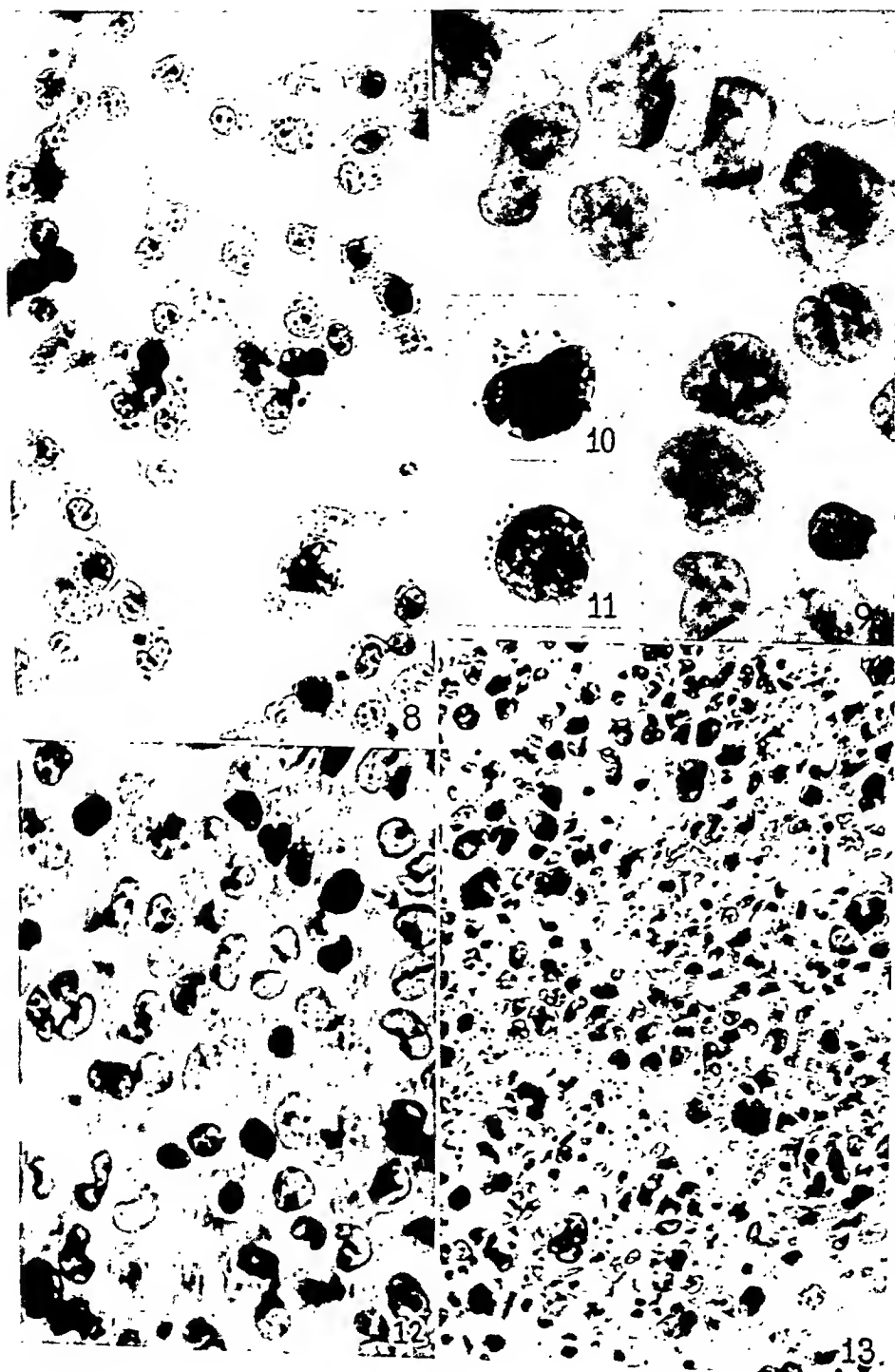
PLATE 8

FIGS. 14 and 15. The malignant cells of human round cell sarcoma or histiocytoma infiltrating the jejunum.   Fig. 14, × 800; Fig. 15, × 1000.

FIG. 16. The malignant cells of human histiocytoma infiltrating the tonsil. × 800.

FIG. 17. The malignant mononuclear cells of transmitted mouse histiocytoma (second passage) infiltrating the liver.   × 800.

PLATE 8
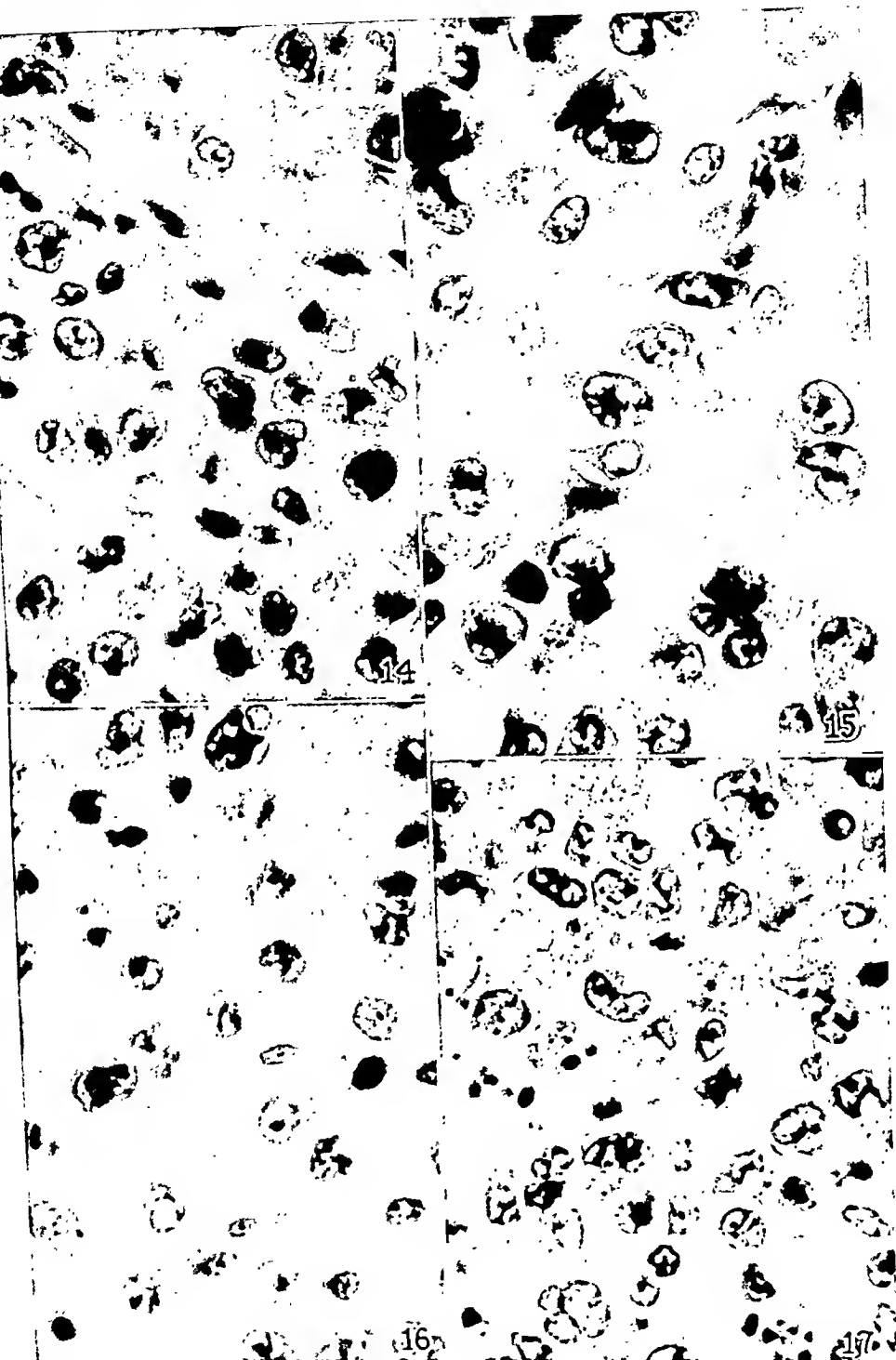
(Furth: Neoplasm of monocytes)

INFECTIOUS MYXOMATOSIS OF RABBITS

Studies of a Soluble Antigen Associated with the Disease

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The occurrence of a soluble, serologically active substance in materials from rabbits with infectious myxomatosis has been reported (1); both serum and extracts of infected skin, obtained from animals acutely ill with the disease and freed from virus by filtration, contain a precipitinogen which reacts specifically with serum of animals recovered from fibroma and myxoma. Study on this soluble substance or substances has been continued in the hope of learning its significance in myxomatosis, *viz.*, what part it plays in the production of immunity to infection and in the excitation of neutralizing, precipitating and agglutinating antibodies. Such an investigation would obviously be simplified if the precipitinogen were free from serologically inert material. The purpose of the present paper is to record the effect of certain physical, chemical, and biological agents on the precipitinogen and to indicate the direction in which purification may be pursued. In addition, observations on the immunological and serological phenomena associated with the partially purified antigen are presented.

Materials and Methods

Filtered Extract of Infected Skin.—Rabbits inoculated intradermally in numerous sites over the backs and flanks with a bacteriologically sterile emulsion of infectious skin are sacrificed, generally on the 6th day, when the resulting lesions are fully developed but not yet necrotic. The skin is macerated in a meat grinder, covered with physiological salt solution, and stored at 0°C. Ether is added to prevent bacterial growth. After periods, varying from several days to several months, the fluid is cleared of large particles and passed through a Seitz filter. Filtration is always slow. The filtrate is a reddish brown, highly viscous solution, rich in the mucoid material typically present in lesions produced by the virus of myxoma.

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Dermal Filtrate.—Another kind of filtrate is obtained, as previously described by Rivers and Ward (1), in the preparation of suspensions of purified elementary bodies of myxoma from dermal pulp of rabbits infected by means of scarification. The infected skin is scraped while covered with dilute disodium phosphate-citric acid buffer solution, pH 7.2, and the resultant suspension, after preliminary horizontal centrifugation, is spun in an angle centrifuge. The supernatant fluid, removed from the sedimented elementary bodies, is passed through a Seitz filter. The filtrate is a clear, slightly yellow fluid.

Filtrate of Serum.—A third source of soluble antigen is the serum of rabbits acutely ill with myxoma as a result of extensive infection of the skin. Serum is separated from blood, obtained by cardiac puncture on the 5th or 6th day after inoculation, and filtered through a Seitz pad.

Immune Sera.—Sera collected from rabbits which had been repeatedly inoculated with myxoma virus after recovery from an infection with fibroma virus (1) have been pooled and used in a dilution of 1:8 for precipitin reactions. Convalescent serum from a rabbit which survived an attack of myxomatosis that resulted from infection by contact was also used; this serum was supplied by Dr. R. F. Parker. The specificity of serological reactions has been controlled by use of sera from normal rabbits or from rabbits recovered from a vaccinal infection.

Precipitin and Agglutinin Reactions.—Precipitin and agglutinin reactions have been conducted as previously described (1): graded dilutions of soluble antigenic material to be tested were mixed with constant amounts of diluted immune or control sera; for the agglutination tests graded dilutions of serum were added to a properly diluted suspension of elementary bodies. All mixtures were incubated in closed racks overnight at 50°C.

EXPERIMENTAL

Filtrates prepared in the manner described are free from virus but contain soluble material or materials which precipitate in the presence of immune sera. Different preparations vary in their content of soluble antigen and in general a lower titer is obtained with dermal filtrate than with filtered extract of skin or with filtered serum. Consequently, most of the work has been conducted on the serologically active material derived from the last two sources. Experience with all three types of filtrate, however, both freshly prepared and after storage, indicates that there is a common or closely related antigen in each.

Effect of Physical, Chemical, and Biological Agents on the Soluble Material

The occurrence of soluble antigen or antigens, separate from infectious agents, has been noted in connection with bacteriophagy (2),

yellow fever (3), vaccinia (4), psittacosis (5), influenza (6), and myxo-
matosis (1). A soluble antigen that is either completely or almost
completely resistant to inactivation at a temperature of 50–56°C. has
been described in all the virus diseases mentioned except myxoma. A
second soluble antigen, which is inactivated at a temperature of 50°C.
is present in filtrates made from tissues infected with vaccine virus.
In view of these facts, an investigation of the stability of the soluble
antigen or antigens of myxoma was indicated and the effect of heat
was examined first.

Small amounts of a filtrate were placed in separate tubes, raised to the tempera-
ture desired, allowed to remain at that temperature in a water bath for different
periods of time, and then cooled rapidly. Frequently, considerable opalescence
appeared in the contents of tubes incubated at relatively high temperatures.
When this occurred, the precipitate was removed before tests were made for the
presence or absence of serologically active material in the supernatant fluids.
In Table I the results of typical experiments with all three types of active filtrate·
are summarized.

It is apparent from Table I that the precipitinogen is usually inac-
tivated by a temperature of 56°C. for 1 hour; at times it may be inac-
tivated in 15 minutes. No heat-stable soluble antigen separable from
the virus has been found in myxoma and in that respect it differs from
other virus diseases studied. This lability renders impossible the
purification of the active principle through coagulation of serologically
inert protein by means of heat.

The range of hydrogen ion concentrations in which the precipitin-
ogen retains its activity was next investigated.

The nature of filtrates derived from macerated skin and from serum is such
that the hydrogen ion concentration cannot be changed readily by the use of
buffer solutions. Adjustment of the pH is accomplished, therefore, by addition
of normal or tenth-normal NaOH and HCl. The accuracy of colorimetric methods
which have been used as routine in the estimation of pH values was controlled on
several occasions by determinations with a glass electrode.

Samples of 5 cc. of filtered serum were placed in separate tubes and adjusted
to pH 3.0, 4.0, 4.5, 5.0, 7.0, and 9.0, respectively, by addition of appropriate
amounts of normal HCl or NaOH solutions. The tubes were stored at +5°C.
for 2 hours, and then spun in an angle centrifuge for 1 hour at 0°C. to remove
the slight precipitates which had formed in the tubes at pH 4.0, 4.5, and 5.0.
The supernatant fluids were readjusted to pH 7.0 and brought to equal volumes
(8.0 cc.) with physiological saline solution. Results are summarized in Table II.

The results recorded in Table II show that activity of the precipitinogen in filtered serum was lost at pH 3.0, and slightly decreased

TABLE I

Effect of Heat on Serological Activity of Soluble Antigen of Myxoma

Type of filtrate	Temp.	Time exposed	Dilution of antigen					
			1:2	1:4	1:8	1:16	1:32	1:64
	°C.							
Extract of skin	37	1 hr.	++++	++++	+++	±	−	
	56	15 min.	++++	++++	±	−	−	
	56	1 hr.	±	−	−	−	−	
		Untreated	++++	+++	++	±	±	
Filtrate of serum	37	1 hr.	±	±	++++	++++	++++	±
	56	15 min.	±	±	−	−	−	−
	56	30 min.	−	−	−	−	−	−
		Untreated	±	+	++++	++++	++++	±
Dermal filtrate	56	1 hr.	−	−	−	−	−	
		Untreated	+++	+++	+++	++	−	

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.

TABLE II

Serological Activity of Filtered Serum, Obtained from Acutely Ill Myxomatous Rabbits, after 4 Hours at Various Hydrogen Ion Concentrations

pH	Dilution of antigen				
	1:4	1:8	1:16	1:32	1:64
3.0	±	−	−	−	−
4.0	++++	++++	++	±	−
4.5	++++	++++	++++	−	−
5.0	++++	++++	++++	++	−
7.0	+	++++	++++	++++	±
9.0	++++	++++	++++	+++	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.

at pH 4.0 and 4.5, but that between pH 5.0 and 9.0 little change occurred. Similar results have been obtained with filtered extracts of

infected skin in which the antigen is only slightly affected by remaining overnight at pH 4.0 at 0°C.; however, a dark brown inactive precipitate formed which could be removed as shown in experiments on purification.

A precipitate which forms between pH 4.0 and 5.0, and which is greatest in amount at about pH 4.5, appears to be different from the one just mentioned. This material is readily soluble when the pH is changed either to below 4.0 or above 5.0, but if it is removed and washed with saline buffered at pH 4.5 only a small part redissolves at pH 7.0. The experiment detailed below and recorded in Table III indicates that at pH 4.5 some active material is precipitated but that the supernatant fluid also retains activity.

One of two 5 cc. samples of filtered extract of infected skin was adjusted to pH 4.0 and the other to pH 4.5 by addition of small amounts of half-normal HCl. A precipitate appeared immediately in each sample but was coarser and heavier at pH 4.5. The precipitates were removed after the mixtures had remained for 2 hours at +5°C. and each was washed 3 times with physiological saline solution buffered at pH 4.0 and 4.5 respectively. The washed sediments were resuspended in physiological saline solution, pH 7.0; only a small part of each sediment went into solution and the residues, which were insoluble, were discarded. The original supernatant fluids containing the components of the filtrate of skin which were soluble at pH 4.0 and 4.5 were restored to pH 7.0 by means of small amounts of half-normal NaOH. Since the final volumes of the four samples were unequal, Table III indicates only approximate titers.

These investigations and others on the behavior of the precipitinogen in acid and alkaline solutions suggest that the isoelectric point of the active material is near pH 4.5. However, experiments reported so far were conducted only in solutions containing normal concentrations of salt; hence, the effect of change in concentration of electrolytes became of interest. A preliminary experiment showed that the specific substance precipitates completely in a half saturated solution of ammonium sulfate. Smaller concentrations of ammonium sulfate have not been effective in completely precipitating the active principle when filtrates are treated at pH 7.0. However, the experiment detailed below shows that 30 per cent saturation of ammonium sulfate at pH 4.5 does completely precipitate it.

100 cc. of filtered extract of infected skin (titer 1:32) which had been partially purified by removal of material insoluble at pH 4.0 and by removal of the albumin

fraction by means of ammonium sulfate were divided into two equal portions; one was adjusted to pH 4.5, the other was kept at pH 7.0. Sufficient saturated ammonium sulfate solution was added to bring the concentration in each portion of filtrate to 30 per cent. A precipitate appeared in both samples immediately, but, after standing at +5°C. overnight, the sample at pH 4.5 was observed to have a heavy precipitate in a yellowish supernatant fluid while that at pH 7.0 had a moderate precipitate in a reddish brown supernatant fluid. Both sediments were removed by centrifugation and dissolved in dilute buffer solution. The redissolved sediments and both supernatant fluids were brought to pH 7.0 and dialyzed overnight against running water. The final volume of each of the supernatant fluids after dialysis was 115 cc. The material precipitated by 30

TABLE III

Serological Activity of Soluble and Insoluble Fractions of Filtered Extract of Infected Skin Obtained at pH 4.0 and 4.5

pH	Fraction	Dilution of antigen				
		Undiluted	1:2	1:4	1:8	1:16
4.0	Soluble	++++	++++	++++	++	±
	Insoluble	−	−	−	−	−
4.5	Soluble	++++	++++	++++	−	−
	Insoluble	++++	+	−	−	−
7.0	Untreated	+++	++++	++++	++++	±

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
See text for preparation and use of fractions in the reactions.

per cent saturation of ammonium sulfate at pH 4.5 was contained in 24.5 cc., while that precipitated at pH 7.0 was in 11 cc.

Results of titration of the four fluids obtained in the above experiment are given in Table IV. Most of the active principle remains soluble in a concentration of 30 per cent saturated ammonium sulfate at pH 7.0, but at pH 4.5 practically all of it is precipitated. This is additional evidence that the isoelectric point of the active principle is near pH 4.5.

It has been observed that the heat-stable antigen of vaccinia (S) remains soluble in 80 per cent alcohol when the reaction of the solution is slightly acid, but is insoluble when the alcoholic solution is neutral (7). A similar behavior on the part of the soluble antigen of

myxoma would facilitate its separation from serologically inactive proteins which are present in all crude preparations and especially in extracts of skin. However, the antigen is completely precipitated in the cold, without inactivation, from serum and from extracts of

TABLE IV

Serological Activity of Soluble and Insoluble Fractions of Extract of Infected Skin Obtained by Treatment with 30 Per Cent Ammonium Sulfate at pH 4.5 and 7.0

pH	Fraction	Dilution of antigen					
		1:2	1:4	1:8	1:16	1:32	1:64
7.0	Insoluble	−	++++	++++	++	−	−
	Soluble	++	++++	++++	++	−	−
4.5	Insoluble	−	±	++++	++++	++++	+
	Soluble	−	−	±	−	−	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
See text for preparation and use of fractions in the reactions.

TABLE V

Serological Activity of Filtered Serum, Obtained from Acutely Ill Myxomatous Rabbits, after Contact with Formaldehyde (U.S.P.) for 18 Hours

Formaldehyde	Dilution of antigen				
	1:2	1:4	1:8	1:16	1:32
per cent					
None	+++	++++	++++	±	−
0.5	+	++++	++++	±	−
1.0	−	++	+	−	−
2.0	−	−	−	−	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
Formaldehyde was removed by dialysis before tests were performed.

infected skin by the addition of 9 volumes of absolute alcohol either at pH 6.0 or at 7.2. Under these conditions the serologically inactive proteins as well as the active principle are precipitated. Acetone also precipitates the active fraction, but no appreciable separation from inert material is effected.

Treatment of the soluble substance with formaldehyde in concentration greater than 0.5 per cent leads to loss of activity (Table V).

The effect on the antigen of a 5 per cent solution of cysteine hydrochloride, which had been brought to pH 7.0, was investigated; this mild reducing agent does not inactivate filtrates after storage overnight at +5°C., nor does its presence prevent inactivation of the antigen by heat.

The effect of a mixture of proteolytic enzymes, namely, commercial trypsin, on the activity of filtered serum and extract of infected skin was investigated. Diminution in the specific serological activity of the filtrates has not followed their treatment with 1 per cent active commercial trypsin which had been previously extracted with petrol ether and ethyl ether while in the dry state.

Partial Purification of Soluble Antigen of Myxoma

With the information recorded above at hand, namely, that the antigen can be partially separated from associated serologically inert material by methods of differential precipitation, based on its solubility at different pH values and in different concentrations of ammonium sulfate, we attempted to purify the soluble antigen in filtrates obtained from serum of infected animals and from extracts of infected skin. An experiment on the purification of the antigen from each type of filtrate will be presented in detail, after which the characteristics of the resulting materials will be described.

85 cc. of filtered serum, which had a precipitin titer of 1:64, were mixed with an equal volume of saturated ammonium sulfate. The precipitate which formed was removed, dissolved in dilute buffer solution, and dialyzed against running water until tests were negative for sulfate ions. The small amount of flocculent precipitate which was present in the dialysate was washed in distilled water; only a part of this was soluble in physiological saline, and, because it was found to be only slightly active, the solution was discarded. Sufficient sodium chloride to make a physiological concentration was dissolved in the fraction containing the soluble dialysate. Then this fraction was adjusted to pH 4.5, after which sufficient ammonium sulfate was added to make a final concentration of 30 per cent. The precipitate which formed was removed after storage for 18 hours at +5°C., dissolved in dilute buffer solution at pH 7.0, and dialyzed overnight against running water. Again a slight precipitate appeared in the dialyzed solution; it was also discarded after it had been shown to possess only slight

serological activity. A precipitin titration carried out with a small amount of the 56 cc. of solution obtained after dialysis gave approximately the same end-point as had the original filtrate of serum from infected animals. The solution was adjusted to pH 8.0 and 1 cc. of a 1 per cent solution of commercial trypsin which had been extracted with petrol ether and ethyl ether was added. After incubation at 37°C. for 1 hour and at +5°C. overnight, the solution was again precipitated at pH 4.5 in a concentration of ammonium sulfate equal to 30 per cent saturation in order to remove as much trypsin as possible. The resultant fine white precipitate was dissolved in dilute buffer solution, pH 7.2, and dialyzed until sulfate ions were no longer detectable; then it was desiccated from the frozen state. 0.5741 gm. of white powder were obtained.

The dry antigen, purified in the manner described, dissolved completely in 6 cc. of distilled water forming an opalescent solution; then sufficient sodium chloride was added to make a physiological solution. The precipitin titer of this solution, calculated on the basis of the above dry weight of partially purified material, was approximately 1:25,000. When a 1:100 solution of this material was examined spectroscopically,[1] absorption bands indicating the presence of tryptophane, tyrosine, and phenylalanine were found; there was no spectroscopic evidence of the presence of nucleic acid. After the solution had been stored at +5°C. for a week, a small amount of a very fine white precipitate appeared and was removed by ultracentrifugation at 20,000 R.P.M. for 20 minutes; the precipitin titer of the supernatant fluid remained the same. The solution was further stored in a frozen state for 3 weeks and then desiccated again. A dry residue weighing 0.4582 gm. was obtained. After still further storage in the dry state at 0°C. for several weeks, a portion of the preparation was removed for study. Two 5 mg. samples were negative for yeast and thymonucleic acid, respectively; a 10 mg. sample gave a delayed, faint but definitely positive Molisch reaction; the dried material contained 15.4 per cent nitrogen.[2] The precipitin titer on an aliquot of the redissolved material was of the order of 1:10,000 (dry weight). Another portion of the 0.4582 gm. of dry residue was removed after additional storage, in a sealed tube, for 2 months at room temperature. The material now failed to react with fibromyxoma serum even when a solution of it contained 1 part dry weight in 750. Moreover, this inert substance no longer showed the solubilities of the original antigen for it now precipitated readily when the pH of the solution was in the neighborhood of 5.4, but remained soluble at pH 4.5.

It is apparent from the record of purification of soluble antigen contained in serum that the method employed resulted in the separation of the serologically active portion from much inactive material.

[1] Spectroscopic examination performed by Dr. G. I. Lavin.
[2] Chemical analyses carried out by Dr. R. J. Dubos.

However, when partially purified, the antigen or antigens obtained from serum are relatively unstable, its activity decreasing under conditions of storage.

The soluble antigen from a filtered extract of infected skin has been refined in the following manner.

1500 cc. of a filtrate of infected skin were adjusted to pH 4.0 by the addition of 150 cc. of normal HCl. The solution became cloudy and was allowed to stand overnight at 0°C. A dark brown insoluble material was removed by centrifugation and discarded. 1540 cc. of clear reddish brown supernatant material were adjusted to pH 7.0 and an equal volume of a saturated solution of ammonium sulfate was added. A heavy precipitate formed immediately and was removed by centrifugation after storage overnight at +5°C. After dialysis the albumin fraction was found to be serologically inactive and was discarded. The globulin fraction was dissolved in dilute phosphate buffer, pH 7.2, and dialyzed against running water overnight. A small amount of insoluble material which was present after dialysis was removed by centrifugation and discarded. The 525 cc. of soluble dialysate were then dried from the frozen state. The desiccated material dissolved completely in 50 cc. of physiological saline solution. The solution was adjusted to pH 4.5 and a saturated solution of ammonium sulfate in amount sufficient to yield a concentration of 30 per cent was added. The precipitate which formed was removed after storage for 18 hours at 0°C. and dissolved in water. After dialysis against running water the resultant solution was clear and dark reddish brown in color. When diluted 1:128, it formed a precipitate with immune serum, a 4-fold increase in the titer, whereas the volume of the final preparation was 1/12 that of the original filtrate. After the partially purified solution had been stored for a short time at 0°C., insoluble material was observed to have formed. This was removed but more formed. However, removal of the sediment caused no appreciable decrease in the titer of this preparation and after standing for 2 months it still gave a precipitin test in a dilution of 1:128.

A method of purification of soluble antigen in filtered extract of infected skin similar to that employed for purification of antigen in filtered serum seems to be effective in separating the serologically active material from some of the associated inactive substances. However, the final preparations of filtrates of skin which we have obtained have always retained some of the dark color of the original fluids, and from this, as well as from the spontaneous precipitation of material without decrease in titer, it is obvious that they are impure. The partially purified antigen from preparations of infected

skin seems to withstand storage better than that in the apparently cleaner preparations from serum.

Serological and Immunological Studies on Rabbits Injected with Partially Purified Antigen from Extract of Infected Skin

Further study of the nature of the soluble substance of myxoma has included an attempt to determine its antigenicity. Antigen from extracts of infected skin, partially purified without tryptic digestion in the manner described above, was injected into normal rabbits and the response of the animals was investigated.

A sample of normal serum was obtained from each of six young adult male rabbits of the New Zealand breed. The animals were then inoculated intra-peritoneally with 2 cc. of a preparation from extracted skin which was known to be non-infectious. The solution had been rendered bacteriologically sterile by Seitz filtration after purification. No untoward reaction developed and during a subsequent 2 week period each animal received a total of 21 cc. of the extract given in six doses. On the 4th day following the last inoculation 10 cc. of blood were taken from each of the rabbits for serological tests. Each of the sera, after inactivation at 56°C. for 30 minutes, gave a precipitin reaction when mixed with the antigen used for immunization. In this instance, dilutions of antisera were tested against constant amounts of antigen; the antibody titers varied between 1:8 and 1:32, but in the majority of the sera they were 1:16. Since precipitins were present in the blood of the rabbits after this short course of treatment, injections were discontinued. 50 cc. of blood were withdrawn from each animal on the 5th day following the last inoculation and the specimens of serum were pooled.

Precipitin Reactions with Anti-Soluble Substance Serum.—The pooled antiserum reacted with partially purified antigen prepared from filtered serum and from extracted skin and also with antigen present in crude preparations of serum, extract of skin, and dermal filtrate. Table VI presents data on such precipitin titrations; the dermal filtrate and filtrate of serum were crude preparations but the antigen from skin had been partially purified. For comparison, the results of titrations with the same antigenic solutions and fibromyxoma serum are included in the table. The pooled anti-soluble substance serum failed to give a precipitate when mixed with any of these sero-logically active solutions that had been heated at 56°C. for 1 hour; moreover, it did not react with vaccinal dermal filtrate known to con-

tain the heat-labile and heat-stable antigens of vaccinia. Thus, the sera appeared to react specifically with the soluble substance of myxoma. Further evidence for the specificity of the reaction was obtained by absorption experiments.

4 to 6 cc. amounts of pooled anti-soluble substance serum were absorbed with crude dermal filtrate, crude filtrate of serum, and refined extract of infected skin, respectively. The ratio of optimal precipitation was initially determined for each antigenic solution by the method of Dean and Webb. Antiserum and antigen were mixed in the proper amounts, incubated at 56°C. for 1 hour and

TABLE VI

Results of Reaction of Fibromyxoma Serum and Anti-Soluble Substance Serum with the Soluble Antigen

Serum	Antigen	Dilution of antigen						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Anti-soluble substance	Dermal filtrate	++++	++++	++++	+	−	−	
Fibromyxoma		++++	++++	+	±	−	−	
Anti-soluble substance	Filtrate of serum	++++	++++	++++	++++	++	−	
Fibromyxoma		±	++++	++++	++++	±	−	
Anti-soluble substance	Extract of skin	++++	++++	++++	++++	++++	++	−
Fibromyxoma		±	+++	++++	++++	±	−	−

All sera used in dilution 1:8.
+'s indicate degree of precipitation.

then overnight at 0°C. Precipitate which formed was removed by centrifugation at 0°C. In several different experiments a single absorption with dermal filtrate or extract of skin was sufficient to remove all or practically all the antibody capable of reacting with the antigen employed for absorption. This was not the case in two experiments when filtrate of serum served as the absorbing antigen; here it was necessary to repeat the process two or three times. Absorbed sera were brought to a dilution of 1:8 of the original serum prior to testing for precipitins.

The results of the experiment presented in Table VII show that absorption of anti-soluble substance serum with dermal filtrate completely removes the antibodies that precipitate in the presence of der-

TABLE VII

Results of Absorption Experiments

Anti-soluble substance serum	Antigen	Dilution of antigen						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Unabsorbed	Dermal filtrate	+++	+++	−	−	−	++	+
	Purified antigen	++	+++	++++	+++++	++	++++	+
	Filtrate of serum	+++	+++++	++	+++++	+++++	++++	−
	" " " heated	−	−	−	−	−	−	−
Absorbed with dermal filtrate	Dermal filtrate	−	−	−	−	−	−	−
	Purified antigen	−	−	−	−	−	−	−
	Filtrate of serum	+++	++	++	+	+	−	−
	" " " heated	−	−	−	−	−	−	−
Absorbed with purified antigen	Dermal filtrate	−	−	−	−	−	−	−
	Purified antigen	−	−	−	−	−	−	−
	Filtrate of serum	++++	+++++	+++	+	+	−	−
	" " " heated	−	−	−	−	−	−	−
Absorbed with filtrate of serum	Dermal filtrate	−	−	−	−	−	−	−
	Purified antigen	−	−	−	−	−	−	−
	Filtrate of serum	−	−	−	−	−	−	−
	" " " heated	−	−	−	−	−	−	−

All sera used in dilution 1:8.

+'s indicate degree of precipitation.

Purified antigen was obtained from extract of skin.

Heated filtrate of serum was held at 56°C. for 1 hour.

The same antigenic solutions were used throughout.

mal filtrate as well as those that react with extract of skin. The same result is obtained by absorption with extract of skin. These absorbed sera, however, still precipitate when mixed with filtrate of serum; moreover, the amount of residual precipitin that reacts with filtrate of serum is appreciable, for its titer is approximately one-fourth that of the unabsorbed serum. As stated above, repeated absorption of anti-soluble substance serum with filtrate of serum was necessary to remove all of the substances that precipitated in the presence of this antigenic solution. Each absorption with filtrate of serum affected the antibodies that precipitated with dermal filtrate and extract of skin to a greater extent than it did those that precipitated with the absorbing antigen. As a result one or two additions of filtrate of serum sufficed to clear the anti-soluble substance serum of precipitins for dermal filtrate or extract of skin but left behind an appreciable amount of antibody that precipitated with filtrate of serum. This previously unrecognized precipitable material encountered in filtrate of serum is also inactivated by heating at 56°C. for 1 hour (Table VII).

The origin and significance of the second precipitable substance, which occurs in relatively large amounts in filtrates of serum and which differs serologically from the soluble antigen demonstrable in all of the three types of filtrates used in this work, has not yet been determined. Whether it is an entirely independent soluble antigen or a substance closely related to the more common soluble substance remains to be seen. It has been found that purification of the common soluble antigen from serum removes or inactivates the second precipitable substance. For example, a sample of serum which in the crude state reacted with anti-soluble substance serum, either unabsorbed or absorbed with dermal filtrate or antigen from skin, after purification reacted only with unabsorbed serum. It should be noted, however, that antibodies against the second precipitable material were induced by injections of partially purified extract of skin, although absorption with similar extracts of skin did not readily remove these antibodies.

Agglutination Reactions with Anti-Soluble Substance Serum.—Antiserum prepared against purified antigen from extract of infected skin agglutinated certain suspensions of elementary bodies of myxoma. The titer of this serum was generally the same as that of the fibro-

myxoma serum used throughout these studies; a myxoma convalescent serum was slightly more potent than either of the other two. Dermal filtrate obtained during the process of purification of these suspensions of agglutinable elementary bodies contained appreciable amounts of precipitinogen that reacted with anti-soluble substance serum and with fibromyxoma serum. Other suspensions of elementary bodies gave lower agglutinin titers with anti-soluble substance serum than with fibromyxoma serum. Indeed, one suspension was not at all agglutinated by anti-soluble substance serum but reacted with fibromyxoma serum diluted 1:128, and it is of interest to note that dermal filtrate obtained from the rabbits furnishing the suspension of elementary bodies failed to precipitate with anti-soluble substance serum and with fibromyxoma serum.

These observations suggest that the soluble substance of myxoma is only one of two or more antigens involved in the agglutination of elementary bodies by immune serum. Confirmation of this was obtained by the use of suspensions of elementary bodies that had been heated at 56°C. for 1 hour. The agglutinin titer of fibromyxoma and myxoma immune serum was usually as high when tested against heated virus as it was when unheated suspensions were used. The character of the agglutination, however, was different; unheated elementary bodies formed large flocks while heated elementary bodies often gave rise to a very fine granular type of agglutination. On the other hand, suspensions of elementary bodies that were agglutinated by anti-soluble substance serum were, after heating, much less agglutinable in the presence of this serum. The variability in the agglutination of different preparations of elementary bodies is not predictable and will be investigated further. However, results so far obtained are adequate for the following statements: elementary bodies of myxoma, separated from crude dermal pulp containing demonstrable amounts of soluble substance, are agglutinated by anti-soluble substance serum and at least some of this agglutination is due to a heat-labile antigen; elementary bodies contain a relatively heat-stable agglutinogen that reacts with antibodies present in fibromyxoma and myxoma immune serum.

Neutralization Tests with Anti-Soluble Substance Serum.—Pooled anti-soluble substance serum used for the precipitin and agglutination

tests described in the preceding sections was tested for the presence of neutralizing antibodies against the virus of myxoma. Equal volumes of diluted virus and undiluted serum were mixed, stored overnight at 0°C., and injected intradermally into each of two rabbits. Suspensions of myxomatous tissue were infective in dilutions of 10^{-5} or 10^{-6}, in different experiments, when mixed with normal or with anti-soluble substance serum. Fibromyxoma serum regularly neutralized 100 infective doses of the virus. These experiments indicate that antibodies against the soluble substance or substances of myxoma are not effective in neutralizing the virus.

Response to Infection by Rabbits That Had Received Injections of Soluble Antigen.—Following the course of injections of partially purified antigen from infected skin, groups of two rabbits each were tested for response to inoculation with the viruses of myxoma, neuromyxoma, and fibroma, respectively. Animals inoculated with the virus of myxomatosis several days after their seventh injection of antigen from skin, developed a typical infection and died on the 8th and 10th days respectively. Several weeks later the four remaining rabbits were given a second course of four injections of partially purified antigen from skin, because the precipitins in their sera had diminished. 5 days after the last injection in this series, precipitin titers in their sera were again equal to or greater than those induced by the first course of treatments. These animals, inoculated with the viruses of neuromyxoma and fibroma, responded in a manner similar to that of normal control animals. Thus, it appears that rabbits injected with non-infectious extract of myxomatous skin were not rendered immune to the virus of myxoma, neuromyxoma, or fibroma, even though their serum contained precipitins against the soluble substance of myxoma and agglutinins for elementary bodies of myxoma.

DISCUSSION

The experiments have shown the soluble antigen of myxoma present in infected skin and in serum of acutely ill rabbits to be a protein. Rabbits receiving intraperitoneal injections of the partially purified antigen from extracts of infected skin readily develop in their sera specific precipitins against the soluble substance. None of the previously studied soluble antigens associated with virus diseases appears

capable, upon injection into animals, of inducing significant resistance to infection or the formation of appreciable amounts of neutralizing antibodies (yellow fever (3), influenza (8), vaccinia (9, 10)); the soluble substance of myxoma behaves in a similar manner.

Anti-soluble substance serum agglutinates elementary bodies of myxoma under certain conditions. However, the soluble substance is only one of several antigens that take part in the agglutination of elementary bodies of myxoma produced by the serum of animals immune to myxoma; at least one other agglutinogen, relatively heat-stable, is present in or on the virus particles. Thus, in respect to multiplicity of antigens, the elementary bodies of myxoma resemble those of vaccinia (11).

The occurrence of agglutinins for elementary bodies of myxoma in the sera of rabbits recovered from fibroma has been considered by Ledingham (12) to be a possible explanation for the ability of these rabbits to survive infection with the virus of myxoma. The present experiments suggest that the rôle of agglutination alone may be a minor one in this phenomenon since rabbits which possessed agglutinins as the result of injections of soluble antigen, succumbed to infection with the virus of myxoma.

A soluble antigen occurs in the serum of animals ill of yellow fever (3), but it is found in the albumin fraction, while that in the serum of myxomatous rabbits accompanies the globulin fraction.

The present experiments suggest that a second soluble specific substance can be identified in materials from myxomatous rabbits. This substance, also heat-labile, differs serologically from the soluble antigen common to dermal filtrate, extract of skin, and serum of acutely ill animals. The relationship of the two soluble substances remains to be determined.

SUMMARY AND CONCLUSIONS

The soluble antigen of myxoma is a heat-labile protein which has an isoelectric point near pH 4.5 and is precipitated from half saturated solutions of ammonium sulfate. It can be partially purified by methods of differential precipitation based on variations in the pH and electrolyte concentration.

Rabbits receiving the labile, soluble substance of myxoma develop

homologous precipitins and their serum agglutinates elementary bodies of myxoma, provided the dermal pulp from which the bodies are obtained contains the soluble substance; neutralizing antibodies do not appear, however, and the animals are not resistant to infection with the virus of myxoma.

Elementary bodies of myxoma appear to have a heat-stable agglutinogen that operates when brought in contact with serum from animals recovered from myxoma, but little, if at all, when in contact with anti-soluble substance serum.

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DETECTION OF THE VIRUS OF POLIOMYELITIS IN THE NOSE AND THROAT AND GASTRO-INTESTINAL TRACT OF HUMAN BEINGS AND MONKEYS*

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The epidemiology of poliomyelitis strongly suggests the contact mode of infection. Experimental evidence furthermore points more specifically to the olfactory portion of the nasal mucous membrane as the portal of entry of the virus into the body. This concept predicates the presence of the virus in the nasal and oral secretions of patients ill with the disease and perhaps also of healthy carriers and contacts. The experimental evidence in this regard, when subjected to rigid criteria, remains sparse; yet in view of the acknowledged difficulties and uncertainties attending the detection of the virus in human material other than that from the central nervous system, we believe that the virus has been recovered with sufficient frequency to serve as strong confirmatory evidence in support of the contact mode of infection.

A review of the literature in search of experiments in which the virus of poliomyelitis was recovered from human and animal sources, exclusive of the central nervous system, has yielded a surprisingly large number of so called takes. We have attempted to tabulate the efforts of the various previous investigators to recover the virus from nasal secretions and from intestinal contents of man and animals in Tables I to IV. The total number of such attempts is probably larger than indicated on these tables, because occasionally only positive findings have been reported and reference made in the text of the paper to other similar attempts, whose outcome was negative.

TABLE I

*Isolation of Poliomyelitis Virus from Nasopharynx of Human Beings**

Author	Stage of disease	At-tempts	Takes	Pas-sage
(1) Landsteiner, Levaditi and Pastia, 1911	Post mortem	1	1	—
(2) Rosenau, Sheppard and Amoss, 1911	Acute	8	0	—
	Convalescent	10	0	—
(3) Kling, Pettersson and Wernstedt, 1912	Acute	12	7	—
	Contact	12	3	—
	Convalescent	31	17	—
	Post mortem	11	7	—
(4) Thomsen, 1912	Post mortem	2	2	1
(20) Kling and Levaditi, 1913	Acute	12	0	—
	Abortive	9	0	—
(5) Kling and Pettersson, 1914	Contact	4†	1	1
(6) Flexner, Clark and Fraser, 1913	Contact	2†	1	1
(7) Lucas and Osgood, 1913	Convalescent	4‡	2	2
(8) Dubois, Neal and Zingher, 1914	Convalescent	1	1	1
(21) Sawyer, 1915	Convalescent	4	0	—
(9) Taylor and Amoss, 1917	Convalescent Abortive	3	2	1 —

* Explanation of terms and symbols used in Tables I to IV inclusive:

Takes = successful infection of animals with human material.

Passage = successful infection of a second animal with cord suspension from the primary take.

0 = an attempted passage with a negative result.

— = no mention of attempt or no attempt was made to pass the infection to another animal.

The numbers indicating the number of attempts are the closest approximations obtainable from the original papers. Frequently reference is made to other attempts without stating the exact number, and numbers in such instances are derived from protocols and the text.

† Pooled.

‡ From the same patient.

TABLE I—*Concluded*

Author	Stage of disease	At-tempts	Takes	Pas-sage
(10) Flexner and Amoss, 1919	Contact ⎱ Acute ⎰ Convalescent⎰	27	0	—
	Post mortem	14	5	—
(11) Kling, 1928	Acute ⎱ Post mortem⎰	84	3	—
(12) Levaditi, Schmultz and Willemin, 1931	Acute	—	1	—
(13) Levaditi and Willemin, 1931	Acute	12	3	0
(14) Frontali, 1932	Acute	8	1	—
(15) Paul and Trask, 1932	Abortive	12	2	2
(16) Kramer, 1935	Healthy carrier	156	1	1
(17) Paul, Trask and Webster, 1935	Acute abortive	27	1	1
	Contacts	14	0	0
(18) Kramer, Sobel, Grossman and Hoskwith, 1936	Convalescent	20	2	2
(19) Stillman and Brodie, 1937	Acute	15	1	1
(22) Harmon, 1937	Acute	20	0	0

Furthermore, although it is quite probable that the number of takes reported by some of these authors, particularly during the last decade, represent true poliomyelitis, this cannot be said to apply to the findings of a number of the earlier reports. When these reports are analyzed according to the criteria which we now believe essential for positive identification of the virus, (a) the production of the typical disease with paralysis in the experimental animal, (b) typical histopathologic changes in the cord, (c) successful passage of the disease to a second monkey, which at autopsy will present the characteristic pathologic changes in its cord,—the number of positive results rapidly dwindles.

In Table I are summarized 535 individual attempts to recover the

TABLE II

Isolation of Poliomyelitis Virus from the Intestinal Tract of Human Beings*

Author	Stage of disease	At-tempts	Takes	Pas-sage
(3) Kling, Pettersson and Wernstedt, 1912	Acute	10	9	—
	Convalescent	30	18	—
	Post mortem	10	9	—
(20) Kling and Levaditi, 1913	Acute	5	1	—
	Abortive	1	0	—
(21) Sawyer, 1915	Convalescent	4	1	1
(8) Dubois, Neal and Zingher, 1914	Acute	1	0	0
(11) Kling, 1928	Post mortem Abortive Carriers Acutely ill	54	5	—
(13) Levaditi and Willemin, 1931	Acute	4	0	0
(22) Harmon, 1937	Acute	20	5	—
(23) Trask, Vignec and Paul, 1938	Acute Convalescent	5	3	3
		(from one child)		

* See explanatory note under Table I.

TABLE III

Isolation of Poliomyelitis Virus from Nasopharynx of Monkeys*

Author	Stage of disease	At-tempts	Takes	Pas-sage
(24) Landsteiner and Levaditi, 1909	Acute	1	1	—
(25) Leiner and von Weisner, 1910	Acute	1	1	—
(26) Flexner and Lewis, 1910	Convalescent	1	1	—
(27) Landsteiner, Danulesco and Levaditi, 1911	Acute	2	2	—
(30) Osgood and Lucas, 1911	Convalescent	2	2	2
(28) Flexner and Clark, 1911	Convalescent	1	1	—
(4) Thomsen, 1912	Acute	2	1	1
(29) Levaditi and Danulesco, 1912	Acute	13	2	—

* See explanatory note under Table I.

virus from the nasopharynx in the human disease; 64 positive takes
are reported, but only 14 passages to second animals are mentioned.
   In Table II are reviewed 144 individual attempts to recover the
virus from patients' feces or from the intestinal contents at post .
mortem.   In only 4 of the 51 takes reported, were the strains passed
on to second animals; three of these being done on the stools of the
same patient (Trask, Vignec and Paul, 23).

TABLE IV

*Isolation of Poliomyelitis Virus from the Intestinal Tract of Monkeys**

Author	Stage of disease	At-tempts	Takes	Pas-sage
(31) Flexner, Clark and Dochez, 1912	2 hrs. after feeding (50 cc. virus)	2	2	—
(32) Clark, Schindler and Roberts, 1930	48 hrs. after feeding concentrated 10 cc.	3†	1	1
	Acutely ill	24	0	—
(33) Kling, 1931	24 and 48 hrs. after feeding	3	1	—
(34) Levaditi, Kling and Lepine, 1931	24 and 48 hrs. after feeding	2	2	—
(35) Clark, Roberts and Preston, 1932	After injecting virus into small intestines	2	2	—
	After feeding 60 cc. for 3 days	8	5	--
	Acutely ill	3	0	--

* See explanatory note under Table I.
† Pooled.

   In Table III are presented the records of 23 attempts to detect the
virus from the upper respiratory tract of monkeys.   11 positive takes
were cited.   In only 3 instances, however, were the strains passed to
a second monkey, and 2 of these were recovered from the washings
of a single animal.
   In Table IV are tabulated 47 attempts to detect the virus in the
intestinal tracts of monkeys.   In 20 instances the feces were obtained
following feeding experiments in which varying amounts of virus

were administered to normal monkeys by mouth or by stomach tube; and in the remaining 27 experiments, the feces and intestinal contents were obtained from acutely ill animals. All 13 positive takes were in animals which had been fed large doses of virus. It will be noted, however, that in no instance was the virus recovered later than 48 hours following the feeding, and that only one passage to a second animal is recorded. We furthermore found no record of recovery of the virus from feces or intestinal contents obtained during the acute stage of the experimental disease.

From these tables, it can be seen that the virus of poliomyelitis has been recovered from the upper respiratory tract of both man and the monkey appreciably oftener than from the gastro-intestinal tract, except when the animals were fed large quantities of virus. It would seem probable, in recognition of the normal physiologic passage of oral and nasal secretions into the gastro-intestinal tract by reflex swallowing and the relative bacterial content of the materials from these two sources, that the small number of successful recoveries of the virus from the latter was perhaps due to the greater difficulties encountered in removing bacterial contaminants from the feces. We believe that the positive data included in these tables are significant and that further studies along these lines are indicated.

The occurrence of poliomyelitis in Brooklyn in the summers of 1935 and 1937 afforded an opportunity to carry on such investigations. In our first communication (18), based on the 1935 cases (included in Table I), we reported the detection of the virus of poliomyelitis in the nasal washings of 2 of a total of 20 children studied during convalescence. In the present paper, which comprises the results of the 1937 occurrence, we have included examination of the stools, as well as the oral and nasal secretions of patients in varying stages of the disease; and we have also studied the nasal washings and intestinal contents from 7 monkeys sacrificed at the height of the disease.

EXPERIMENTAL

Except for the modifications necessary for the handling of the fecal material and intestinal contents, the procedures employed were essentially those outlined in our previous communication (18).

Collection of Human Nasal Washings.—With the patient lying prone with his head turned to one side, a soft rubber catheter was inserted 1 to 2 inches into one nostril compressed against the catheter to prevent leakage. 50 to 100 cc. of sterile distilled water were gently introduced by means of a large syringe. The return fluid from the opposite nostril and the mouth was caught in a sterile pus basin, and then transferred to a sterile bottle.

Collection of Human Fecal Material.—Fecal material was obtained on the same day as the nasal washings. If the feces were from a spontaneous bowel movement, 100 cc. of sterile distilled water were added; if an enema were given, 100 cc. of sterile distilled water were employed. The feces were then transferred to a 250 cc. sterile centrifuge bottle and shaken vigorously in an eccentric shaker for 1 hour. The well macerated material was then centrifuged at 1250 R.P.M. for 1 hour and the supernatant fluid, usually quite opalescent, was drawn off into another sterile centrifuge bottle.

Collection of Nasal Washings and Tissue from Monkeys.—The animals employed in these experiments were infected either by the intranasal route with the Armstrong strain of virus, or by the intracerebral route with our stock laboratory VM strain.

Nasal washings were obtained at the height of the disease. The animal was quickly sacrificed with ether anesthesia; its face was washed with 5 per cent phenol followed by ether; and with the head held downward over a pus basin, the same procedure was used as was employed for obtaining nasal washings from patients. About 40 cc. of sterile distilled water were used. The margin of the nostrils was then cut, and by means of a fine curette the nasal cavity and posterior pharynx were carefully curetted. The resulting tissue, consisting of the mucous membrane, adenoid tissue, cartilage and some bone fragments, was thoroughly ground with sterile sand in a mortar and added to the nasal washing. This mixture of washing and ground tissue was shaken for 30 minutes in an eccentric mechanical shaker and centrifuged at 1250 R.P.M. for a half hour. The supernatant was poured off into a sterile Erlenmeyer flask.

Collection of Fecal Material from Monkeys.—Immediately after the nasal washing and curetting was completed, the abdominal cavity of the animal was opened and segments of the intestine were tied off. One ligature was applied at the pyloric end of the stomach, two ligatures at the junction of the ileum and cecum, and a single ligature at the anal end of the rectum. The intestines were then dissected away from the omentum, removed from the abdominal cavity and placed on sterile towels or in sterile Petri dishes. The single length of gut was then divided between the second pair of ligatures, resulting in two segments of gut; the first comprising the pylorus, the jejunum and ileum, and the second including the colon, the sigmoid and the rectum. The ligatures were then removed and the contents of each section of gut squeezed into separate containers. 50 cc. of sterile distilled water were then introduced into each segment and, by means of gentle massage, forced through the gut and added to the corresponding intestinal

contents or fecal material. Each section of gut was then slit longitudinally, exposing the mucous membranes, and these were gently curetted. The scrapings were added to the corresponding container. The combined material from each section of gut was placed in 250 cc. centrifuge bottles and shaken vigorously in an eccentric shaker for 1 hour and centrifuged for 30 minutes at 1250 R.P.M. The supernatant liquid was poured off into sterile centrifuge bottles.

Sterilization of Washings and Fecal Material.—The method described by us in a previous communication (18) and recently successfully employed by Trask *et al.* (23) was used in removing bacterial contaminants from human and monkey material. From 10 to 20 per cent of anesthetic ether was added to the flask containing nasal washings, and approximately 40 per cent ether was added to the supernatant material obtained from the intestinal tract. The flasks were tightly
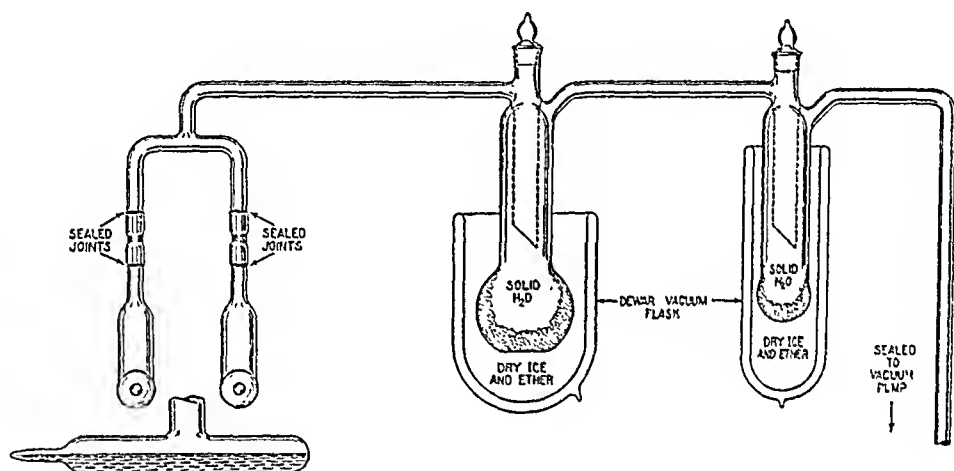
FIG. 1

stoppered and shaken mechanically for from 30 minutes to an hour and left overnight in the ice chest.

Concentration of Materials.—The following morning the materials were recentrifuged and the aqueous portions were transferred by sterile technique to a specially constructed sterile container and attached to the vacuum system (see Fig. 1). The material in each container was frozen solid by immersion in CO_2-acetone mixtures, and the vacuum was started. In 6 to 8 hours the material was concentrated to from 2 to 5 cc., depending on the original volume (50 to 100 cc.).

Although we had previously established (18) that the virus would survive the manipulation involved in our procedure, we again included 3 specimen runs at the beginning of our concentrations. Each of these specimens consisted of 75 cc. of sterile distilled water to which had been added 0.5 cc. of a 5 per cent suspension of our stock VM virus. 10 per cent of anesthetic ether was then added, the mixture well shaken and left overnight in the refrigerator. The following morning

the specimens were placed in the special containers, attached to the desiccating outfit, then frozen and desiccated. All 3 monkeys (Nos. 3-38, 3-39 and 3-46, Table V), inoculated with concentrations from these specimens, succumbed to the experimental disease in from 6 to 10 days.

Inoculation of the Concentrates.—The concentrates were removed from the special containers by cutting the constricted end of the tube and withdrawing the material through this opening with a sterile pipette. 1 to 2 cc. of sterile distilled water were usually added to the emptied container and the walls of the tube were gently rubbed with either a pipette or a glass rod to release any adherent material, which was then added to the concentrate. The fecal concentrates had to be recentrifuged to throw down the heavy particles which appeared during the concentration, and the supernatant liquid was used for inoculation. From 2 to 4 cc. of each concentrate were inoculated intracerebrally into a fresh monkey, and the remainder of the material was inoculated intraperitoneally into the same animal.

RESULTS

Nasal washings and stool or intestinal contents were obtained from 20 patients in varying stages of convalescence, and from 7 monkeys during the height of the disease. 5 strains of virus were recovered, 4 strains from 3 patients and 1 strain from a monkey. The pertinent data are tabulated in Tables V and VI.

The first strain was isolated from the feces of a 14 year old child (S.W.), 7 days after the onset of the illness. A test animal (3-48) inoculated on Aug. 28, was paralyzed on Sept. 17. Histologically, the cord showed typical poliomyelitis. A passage from the cord of this animal intracerebrally into monkey 3-77 on Sept. 17 resulted in paralysis and prostration Sept. 23. Histologically the cord showed typical poliomyelitis.

The second human strain was isolated from the nasal washings of a 6½ year old child (D.E.), 5 days after the onset of illness. The test animal (3-50) was inoculated on Aug. 31, and developed weakness of both lower extremities and the left arm on Sept. 7. It was found dead on the morning of Sept. 8. A cord suspension from this animal inoculated on the same day into monkey 3-68, resulted in paralysis on Sept. 21. Both animals presented typical histologic findings in their cords.

The third and fourth human strains were recovered from a 2½ year old boy (J.C.), 9 days after the onset of the illness. One strain was obtained from the nasopharynx and the other from the feces. The 2 test animals (3-55 and 3-56, Table V) inoculated on Sept. 2 with the concentrates from these sources showed temperature and paralysis on the 11th. Histologic examination of sections from the cords of both these animals showed typical poliomyelitis. Intracerebral inoculations of emulsions of the cords on Sept. 11 into 2 fresh test animals (3-67

TABLE V

Outcome of Inoculations of Concentrates from Human Sources

(Nasal Washings and Feces)

Case	Patient	Age	Date of onset 1937	Date of washings 1937	Time from onset to washings days	Date of inoculation 1937	Source of concentrates	Cultures*	Amount of inoculum cc.	Monkey No.	Fever	Symptoms	Post mortem and histological findings	Passage
													Outcome	
1	S. W.	14 yrs.	8/21	8/27	7	8/28	N†	16 colonies	4	3-47	9/3, 100.2° 9/4, 105.2°	None		M. 3-77, 9/17 i...lated. 9/23 trated. Hist. cal poliomyelit...
							F†	Sterile	4	3-48	None	Weakness of all extremities. Sacrificed	Typical poliomyelitis	
2	A. D.	17	8/20	8/30	10	8/31	N	Not done	4	3-49	None	None		
							F	" "	3	3-52	"	"		
3	D. C.	7 mos.	8/19	8/27	8	8/31	N	Sterile	4	3-51	"	"		
							F	34 colonies	4	3-54	"	Lethargic refused to climb. 9/7 died	Brain abscess. Organisms recovered	
4	D. E.	6 yrs.	8/24	8/28	5	8/31	N	Sterile	3	3-50	9/5, 105° 9/7, 101°	Generalized weakness. 9/8 found dead	Typical poliomyelitis	M. 3-68, 9/8 i...lated. 9/21 trated. Hist. ical poliomyel...
							F	50 colonies	4	3-53	None	Animal in stupor, crouched in cage. 9/1 found dead	No brain abscess	

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5	J. C.	2½	8/25	9/2	9	9/3	N	Not done	4	3-55	9/10, 106° 9/11, 106°	9/11 upper limbs involved. Sacrificed	Typical poliomyelitis	M. 3-69, 9/11 inoculated. 9/21 reinoculated. 10/6 reinoculated. 10/11 prostrated. Hist. typical poliomyelitis.
6	M. O.	9	8/25	9/2	8	9/3	N	Sterile	4	3-56	9/10, 106°	9/11 right upper weak. Sacrificed	"	M. 3-67, 9/11 inoculated. 9/22 prostrated. Hist. typical poliomyelitis.
							F	100 colonies	4	3-60	None	None		
									4	3-62	"	Animal drowsy for 2 days. Recovered		
7	M. F.	6	8/28	9/2	10	9/3	N	19 colonies	4	3-59	"	9/17 generalized weakness. Recovered		
							F	8 colonies	4	3-57	"	None		
8	E. H.	4	8/22	9/2	11	9/3	N	Not done	4	3-58	"	9/4 weakness of upper limbs. 9/7 found dead	No brain abscess	
							F	"	4	3-61	"	"		
9	C. J.	17	8/30	9/7	9	9/8	N	Not done	4	3-63	None	None	Suggestive poliomyelitis	
							F	"	4	3-64	9/21, 106.8° 9/22, 106.4°	Upper left limb paralyzed. Sacrificed		
10	R. L.	8	9/4	9/12	8	9/13	N	"	4	3-66	9/17, 100.4° 9/18, 101.5° 9/25, 106°	None		M. 3-89, 9/23 inoculated. 10/6 reinoculated. 10/20 reinoculated. No take
							F	"	4	3-65		9/26 generalized weakness. Recovered		

* 0.5 cc. to 1 cc. of concentrates used for cultures. The numerals indicate colonies on pour plate.

† N, nasopharynx. F, fecal material.

TABLE V—*Concluded*

Case	Patient	Age (yrs)	Date of onset (1937)	Date of washings (1937)	Time from onset to washings (days)	Date of inoculation (1937)	Source of concentrates	Cultures*	Amount of inoculum (cc.)	Monkey No.	Outcome			
											Fever	Symptoms	Post mortem and histological findings	Passage
11	S. S.	7	9/11	9/14	4	9/15	N	Sterile	4	3-71	None	9/23 left upper and lower paralyzed	Typical cord lesions	M. 3-86, 9/23 inoculated. 10/16 reinoculated. 10/20 reinoculated. No take
							F	100 colonies	3	3-72	"	9/16 animal lethargic. Died	No brain abscess	
12	M. F.	3	9/2	9/14	12	9/16	N	Sterile	4	3-73	"		Brain abscess. Organisms recovered. Lesions not characteristic	
							F	4 colonies	4	3-74	"	9/22 animal in stupor. 9/23 died		
13	S. D.	2	9/9	9/15	6	9/17	N	Sterile	4	3-76	"	None	No gross brain abscess	
							F	47 colonies	4	3-75	"	9/18 drowsy. Refused to climb. All limbs weak. 9/22 died		
14	J. C.	8	9/11	9/16	6	9/18	N	Sterile	4	3-79	"	None	No gross brain abscess	
							F	"	4	3-78	"	"		
15	R. D.	3	9/14	9/19	6	9/22	N	"	4	3-81	10/16, 104.4° 10/18, 99.8° 10/19, 101.6°	Generalized weakness. 9/22 died	Tuberculosis	
							F	60 colonies	3	3-80	None	9/23 lethargic. Died	No brain abscess. Acute suppurative meningitis	

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No.	Name						N/F	Sterile	cc	Monkey	Temperature	Symptoms	Outcome
16	G. A.	10	9/16	9/21	6	9/23	N	Sterile	4	3-82	None	None	No brain abscess
							F	"	3	3-83	9/26, 100°	9/23 crouched in cage. Drowsy. Generalized weakness. 9/27 died	
17	M. H.	21	9/14	9/22	9	9/24	N	"	4	3-84	None	None	Acute suppurative meningitis
							F	"	3	3-85	9/25, 100°	9/25 generalized weakness. Lethargic. Died	
18	M. P.	3	9/11	9/23	10	9/25	N	"	4	3-87	9/29, 105° 9/30, 101°	None	
							F	"	2	3-88	9/30, 101° 10/1, 105.6°	Tremor for 4 days after inoculation. Disinclination to climb. Recovered	
19	E. B.	7	9/21	9/24	4	9/25	N	"	4	3-90	9/28, 105° 10/1, 101°	None	
							F	"	2	3-91	None	"	
20 J. C. (repeat)		21	8/25	10/3	41	10/4	N	"	4	3-95	"	"	
							F	"	3	3-96	"	"	

Controls

Monkey No.	Date of inoculation 1937	Type and amount of inoculum	Outcome
3-38	8/21	0.5 cc. of a 5 per cent cord suspension diluted with 75 cc. of sterile distilled water, frozen and concentrated *in vacuo* to about 4 cc.	Poliomyelitis 8/31/37
3-39	8/26		Poliomyelitis 9/2/37
3-46	8/26		Poliomyelitis 9/1/37

TABLE VI

Outcome of Inoculations of Concentrates from Animal Sources
(Nasopharynx and Intestines)

Acutely ill monkey	Date of washings	Date of inoculation	Source and amount of inoculum	Cultures*	Monkey No.	Outcome			
						Fever	Symptoms	Histological findings	Passage
	1937	*1937*							
2-75	9/27	9/29	Nasal washing 2 cc.	Sterile	3-92	None	None		
			U. intest. seg.† 2 cc.	"	3-93	10/16, 105.8° 10/18, 101.6°	"		
			L. intest. seg.‡	"	3-94	10/19, 106.4°	Animal excited. Recovered		
8-6	10/9	10/11	Nasal washing 4 cc.	6 col.	4-03	None	None		
			U. intest. seg. 3 cc.	23 "	4-01	"	Generalized weakness. 10/17 died	Tuberculosis	
			L. intest. seg. 3 cc.	17 "	4-02	10/19, 104.8°	Limbs weak. 10/19 died	Questionable polio	—
2-7	10/10	10/12	Nasal washing 3 cc.	Sterile	4-05	10/25, 104.8° 10/26, 101.8°	Generalized weakness. No paral. Recovered		
			U. intest. seg. 3 cc.	2 col.	3-70	None	None		
			L. intest. seg. 3 cc.	25 "	4-04	"	"		
3-52	10/13	10/15	Nasal washing 4 cc.	Sterile	4-07	11/5, 105.8°	Lower limbs weak. Recovered		
			U. intest. seg. 3 cc.	"	4-06	10/18, 106.4° 10/19, 106.8°	Continued high temp. 11/21 prostrated	Suggestive polio	M. 4-09, 11/21 inoculated. 12/19 paralyzed. Hist. typical polio
			L. intest. seg. 3 cc.	8 col.	4-08	11/3, 106° 11/5, 106°	Generalized weakness. Recovered		
3-99	11/21	11/23	Nasal washing 3 cc.	Sterile	4-10	12/29, 104.2°	12/10 lethargic, refused to climb. 12/29 limbs weak. 12/30 died	Questionable polio	—

* 0.5 cc. to 1 cc. of concentrates for cultures. The numerals indicate colonies on pour plate.

† Upper intestinal segment.

‡ Lower intestinal segment.

TABLE VI—*Concluded*

Acutely ill monkey	Date of washings	Date of inoculation	Source and amount of inoculum	Cultures*	Monkey No.	Outcome			
						Fever	Symptoms	Histological findings	Passage
3-99 (con-tin-ued)	1937	1937	U. intest. seg. 3 cc.	"	4-11	None	None		
			L. intest. seg. 3 cc.	"	4-12	12/2, 105°	Generalized weakness. Recovered		
4-00	11/25	11/27	Nasal washing 2 cc.	Sterile	4-13	None	None		
			U. intest. seg. 3 cc.	49 col.	4-14	12/1, 105°	12/12 general-ized weakness. 12/14 died	Question-able polio	—
			L. intest. seg. 3 cc.	Ster-ile	4-15	None	None		
3-94	11/25	11/25	Nasal washing 2 cc.	"	4-18	"	"		
			U. intest. seg. 3 cc.	"	4-16	"	12/12 drowsy. Died	No gross ab-scess. Question-able polio	—
			L. intest. seg. 3 cc.	"	4-17	"	None		

and 3-69), resulted in the typical disease in both animals. Monkey 3-67 inoculated with a suspension of the cord from the primary take of fecal origin succumbed in 11 days. Monkey 3-69 inoculated with a suspension of cord from the primary take of the nasal washings succumbed after the third reinoculation of heavy cord suspensions (10, 20 and 20 per cent respectively), the second and third inoculations being given at 2 week intervals, and the animal succumbing 5 days after the third inoculation.

As is indicated in Table V, a number of additional monkeys inoculated with concentrates from human sources presented suggestive elevations in temperature or showed some weakness. One such animal (3-71), sacrificed when it developed paralysis, showed what appeared to be a typical histologic picture of poliomyelitis, yet we were unsuccessful in transmitting the disease to a second monkey, even after repeated reinoculation with heavy suspension of cord. A second monkey (3-64) showed elevation in temperature and some weakness

in the right leg and presented suggestive, though not typical histologic findings of poliomyelitis. We were unable to obtain a successful passage into a second monkey.

The fifth and only strain of virus recovered from the experimental animal came from the concentrate of the material from the upper intestinal segment of monkey 3-52, Table VI, sacrificed at the height of the disease. Monkey 4-06, Table VI, was inoculated with the concentrate from monkey 3-52 on Oct. 15. 3 days later, the animal showed temperature elevation and was prostate on the 21st. The histologic findings were compatible but not typical of poliomyelitis. 2 cc. of a 20 per cent suspension of cord were inoculated into monkey 4-09 and resulted in the experimental disease after a prolonged incubation period. Histologic examination showed typical poliomyelitic changes in its cord.

The remaining 2 concentrates from monkey 3-52 produced symptoms suggestive of the experimental disease in the respective test animals. Both monkey 4-07, which was inoculated with the concentrate of the nasal washing, and monkey 4-08, which received concentrate from the lower intestinal segment, showed elevation in temperature and some suggestive weakness, but no extensive involvement of any of the extremities. Both these animals recovered without residual paralysis.

Bacteriology of Concentrates.—30 of the 40 concentrates from human material, and all of the 21 concentrates from the animal material, were cultured on pour plates, broth in Dunham tubes, and blood plates. 0.5 to 1 cc. of the sediment from each concentrate was used for culture. Since our primary interest was to determine whether or not the concentrates were sterile, no special effort was made to identify the organisms beyond morphology and reaction to Gram stain. The organisms were usually Gram-negative rods, presumably colon bacilli. In one instance a Gram-positive coccus was found, and in two instances yeasts were contaminants.

It will be noted in Table V that of the 15 cultures from the concentrates of the human nasal material, 2 were slightly contaminated, while the remaining 13 were sterile. Of the 15 cultures from the fecal concentrates, 8 were found to contain bacteria.

Of the 21 cultures obtained from animal sources, 7 were slightly contaminated; of these one was a nasal concentrate, the remaining 6 from intestinal contents.

Fifteen animals died from causes other than poliomyelitis; 2 died from tuberculosis, 2 from brain abscesses from which positive cultures

of organisms were recovered. 7 animals, all presenting a similar clinical picture, died from 1 to 5 days following inoculation. These animals apparently never recovered from the inoculation: they appeared lethargic, crouched in their cages with drooped heads, responded poorly to stimuli, and finally died. It is furthermore interesting to note that these animals had all been inoculated with concentrates from intestinal contents. It was our impression that the fecal concentrates contained some toxic factor, as has been suggested by Toomey (36) and others.

It can furthermore be seen that 5 animals which had been inoculated with contaminated concentrates survived. Indeed, one animal (3-62) survived an intracerebral inoculation of a heavily contaminated fecal concentrate, which showed more than 100 colonies on the pour plate.

SUMMARY AND COMMENT

Five strains of virus were recovered from nasal washings and feces. Four strains were of human origin, the fifth strain came from a monkey sacrificed at the height of the disease. Of the four human strains the first was isolated from the feces of a 14 year old child 7 days after the onset of illness. The second strain was from the nasal washings of a $6\frac{1}{2}$ year old child, 5 days after the onset of illness. The third and fourth strains were recovered from the same patient, a $2\frac{1}{2}$ year old child, 9 days after the onset of illness. One of these strains was obtained from nasopharyngeal washings and the other from the feces. The single monkey strain was isolated from the upper intestinal segment and appears to be the only instance of its isolation from this source in the literature.

We believe that the detection of the virus in the nasal washings of two additional patients during convalescence lends further support to the belief that the virus of poliomyelitis is spread by human contact.

Furthermore, the recovery of the virus from the gastro-intestinal tract with as great or greater frequency as from the upper respiratory tract, need not, it appears to us, alter our concept of the mode of entrance of the virus into the body, namely, by way of the upper respiratory tract. If the presence of the virus is conceded, then a

consideration of the physiologic passage of nasal and oral secretions into the gastro-intestinal tract by reflex swallowing would serve to explain adequately the presence of the virus in those organs. It might even be further predicated that since the gastro-intestinal tract functions as a temporary reservoir for secretions from the upper respiratory tract, the gut should, after a time, contain the virus in higher concentration than any single sample of secretion obtained from the upper respiratory tract by nasal washing. It appears to us that failures to detect the virus in the gastro-intestinal tract are perhaps more indicative of inadequate procedures for its detection than of its absence.

The recovery of the virus from the feces 7 and 9 days after the onset of illness takes on added significance. It indicates first, that the virus withstands the gastric acidity which under normal physiological conditions tends to keep gastric contents relatively free of bacteria. It further suggests that improper disposal of feces from patients with poliomyelitis may have serious public health consequences, particularly in smaller communities where inadequate sewage disposal may result in contamination of surrounding beaches or even local water systems.

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A SPREADING FACTOR IN CERTAIN SNAKE VENOMS AND ITS RELATION TO THEIR MODE OF ACTION

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The present investigation was undertaken to determine whether the venom of poisonous snakes contained a factor capable of increasing tissue and blood capillary permeability. A preliminary note on this subject has been published (1).

Substances which effect an increase in connective tissue and capillary permeability have been extracted from a number of biological sources. In normal mammalian tissues, the testis is particularly rich in this spreading factor (2). It has also been demonstrated in malignant tissues (3), in leech extracts (4), and in certain bacteria (5). The elaboration of such substances by certain species of bacteria is correlated with their ability to spread through or invade tissues, or to become rapidly disseminated *via* the blood stream. Thus the association of such spreading factors with noxious agents of one type or another tends to alter considerably their spread through the tissues, and eventually through the body as a whole. So doing, it may in the one instance result in an enhancement of a reaction, and in another act to diminish it, according to the factors involved amongst which are the nature of the spreading agent, and the native ability of the host to deal with it (6).

It is obvious that the presence of a spreading factor in snake venom would have an effect upon the sequence of events following introduction of it. In the present studies we have investigated the spreading factor content of the venom from several species of venomous snakes, of extracts of the supralabial glands of two species of harmless snakes, and of toad venom. In addition experiments are described which were aimed to differentiate the spreading factor from the toxic factor in snake venoms.

Materials and Methods

The Snake Venoms.—Venom from 9 species of snakes was employed. In some instances fresh venom was available, while in others the dried preparations, either

purchased in the open market or obtained from foreign countries, was used.[1] The family and species of the snakes, and the original state of the venom, are indicated in Table I, together with the dilutions prepared for animal injection. Extracts of the supralabial glands from 2 species of harmless snakes were prepared, using 1 gm. of fresh gland and 9 cc. of saline, with subsequent dilution. The dried venoms were dissolved in distilled water to restore the original volume of the fresh secretion. The amount of water to be added was determined by the figures published by Noguchi (7). Subsequent dilutions were made with the addition of 0.9 per cent NaCl solution. When fresh venom was used it was diluted with 0.9 per cent NaCl solution.

Toad Venom.—Venom from 6 species of toads was used. These preparations represented the dried secretions of the skin glands. The names of the species will be found in the corresponding section. For use in these experiments, the dry venom was ground in a mortar with 0.9 per cent NaCl, and finally made up in the proportion of 1.0 gm. of dry venom to 49 cc. of saline solution. This suspension was centrifuged and the supernatant fluid used for injection.

Infectious Agents.—For testing the effect of the venoms on infections, vaccine virus, *Staphylococcus aureus*, and the bacillus of mouse typhoid II were used. The Levaditi strain of neuro-virus was secured from an infected rabbit testicle and prepared by the following method. 5 days after an intratesticular inoculation of neuro-virus suspension the animals were sacrificed, the testes removed and ground with sterile sand in 0.9 per cent saline (1 gm. testes to 9 cc. saline). After centrifugation, the supernatant fluid was diluted in various proportions for the test inoculations, as shown in Table III. The bacterial suspensions were the product of 24 hours growth on plain agar shaken up with 10 cc. of saline. Dilutions of the freshly prepared virus or bacterial suspensions provided the graded series of doses used in the tests.

Testing of the Venoms.—0.5 cc. of each of the venom dilutions was mixed with 0.25 cc. of diluted India ink (1:2) and injected into one flank of a rabbit. On the opposite side, a similar series was injected, into the corresponding locations, but using 0.25 cc. of saline solution in place of the ink. This was done so as to be able to judge the extent of the spread of the injected material by aid of the India ink on one side, and the severity of the lesion produced by the different dilutions of venom uncomplicated by the presence of the ink on the other side.[2] In addition

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[1] We are indebted to Dr. C. H. Kellaway of the Albert and Eliza Hall Institute, Melbourne, Australia, and Dr. C. Picado of the San Juan Hospital, San José, Costa Rica, for supplying several of the dried venoms. The toad venoms were kindly supplied by Dr. G. H. A. Clowes and Dr. K. K. Chen of the Lilly Research Laboratories.

[2] This proved to be a wise precaution for it was noted that the lesions produced by the ink mixtures were smaller and less severe than those produced by the same dilutions of the venom without the ink. Probably some of the spreading factor is absorbed by the carbon particles, as demonstrated in other instances by Favilli and McClean (8).

a control injection was made consisting of 0.5 cc. of saline plus 0.25 cc. of ink.   As a rule no more than 5 dilutions of venom were tested on the same animal.

The area of epidermis beneath which the ink particles had spread was measured at the end of 24 hours, and recorded in square centimeters.   The character of the lesions produced was graded from extremely pronounced through intermediate stages to very mild.   The extremely pronounced lesions were those which were very hemorrhagic, edematous, and necrotic.   The very mild lesions were those which showed only erythema, which disappeared in 24 to 48 hours.   The intermediate classifications were represented as gradations between these extremes. Essentially the same technique and nomenclature were used when testing the effect of the addition of venoms to bacterial and viral suspensions, injected intradermally.

The Relationship between the Spreading Factor and the Local and General Toxicity of Snake Venom

In a preliminary survey we studied the effect on the rabbit of the fresh or dry venoms of 9 species of poisonous snakes as compared with the effect of extracts of supralabial glands of 2 species of harmless snakes.

Experiment.—Progressive saline dilutions of the venom or of the supralabial gland extract were injected intradermally in one side of rabbits in the amount of 0.5 cc. mixed with 0.25 cc. of India ink so as to be able to judge the extent of spreading.   The same injections were repeated in the other side using saline solution instead of India ink.   Each venom was tried on a separate rabbit.   Details and results of the tests are given in Table I.

It is apparent from Table I that all the snake poisons have a more or less marked spreading power, and that extracts from the supralabial glands of harmless snakes do not.   The bleb formed by the injection of the venoms flattens out immediately and the injected material spreads through the skin, causing a hemorrhagic and edematous lesion.   It is also seen that the lesions produced by venoms from the Viperidae are larger and more severe than those produced by the venoms of the Colubridae family.   The fact that 6 out of 10 of the rabbits injected with venoms of the latter group died within 15 hours, whereas none of those injected with the venoms of the Viperidae died, emphasizes the distinction between the locus of action of these two groups of venoms, in agreement with the early work of·Flexner and of Noguchi (7) who found that venoms from the Viperidae (rattlesnakes) contain large amounts of locally acting toxins, and compara-

TABLE I

Spreading Power and Local and General Toxic Power of the Venoms and Supralabial Gland Extracts of Various Snakes

(Results after 24 Hours)

Type of snake	State of secretion and snake species	Area of spread and severity of lesion in rabbit skin injected with 0.50 cc. venom or extract plus 0.25 cc. India ink or saline					Spreading of 0.50 cc. saline plus 0.25 cc. India ink dilution (control)	Number of rabbits injected	Number of rabbits killed by venom
		Dilutions of snake venom							
		1:100	1:1000	1:10,000	1:100,100	1:1,000,000			
Poisonous		sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.		
Viperidae crotalinae	Dry *Crotalus adamanteus* venom (diamond rattlesnake)	95.0 Very pronounced	35.0 Mod. pronounced	19.6 Mod. pronounced	12.8 Mild	7.6 Very mild	6.3	5	0
	Dry *Crotalus terrificus durissus* venom (rattlesnake)	72.0 Very pronounced	30.0 Pronounced	18.2 Mod. pronounced	8.1 Mild	6.0 Very mild	5.0	1	0
	Fresh *Crotalus atrox* venom (western rattlesnake)	70.0 Ext. pronounced	30.0 Very pronounced	19.9 Pronounced	6.2 Mod. pronounced	3.2 Mild	2.6	2	0
	Dry *Ancistrodon piscivorus* venom (water moccasin)	69.0 Pronounced	20.0 Mod. pronounced	19.8 Mod. pronounced	7.0 Very mild	7.0 No lesion	5.6	1	0
	Fresh *Ancistrodon piscivorus* venom (water moccasin)	50.0 Pronounced	15.1 Mild	8.0 Very mild	7.0 No lesion	7.4 No lesion	7.6	1	0
Colubridae proteroglypha	Fresh *Elaps fulvius* venom (coral snake)	72.6 Mod. pronounced	18.6 Mild	12.9 Very mild	12.4 Very mild	8.0 Very mild	8.4	1	1 (15 hrs.)
	Dry *Denisonia superba* venom (superb snake)	46.0 Mod. pronounced	20.0 Mild	14.7 Very mild	8.7 Very mild	6.7 No lesion	4.9	3	1 (15 hrs.)
	Dry *Naja tripudians* venom (cobra)	11.5 Mod. pronounced	10.5 Mild	10.0 Very mild	9.5 Very mild	8.5 No lesion	6.0	2	1 (2 hrs.)
	Dry *Acanthophis antarticus* venom (death adder)		14.5 Very mild	10.9 Very mild	7.3 No lesion	6.1 No lesion	7.2	3	2 (5 hrs.)
	Dry *Notechis scutatus* venom (black tiger)			Some spreading detected. No lesions produced				1	1 (2 min.)
Harmless	Fresh supralabial gland *Pityophis cateniferis* (pine snake)	5.5 No lesion	5.1 No lesion				5.1	1	0
	Fresh supralabial gland *Elaphe quadrivittata* (chicken snake)	10.0	5.0				5.0	1	0

tively small amounts of neurotoxins, whereas the reverse is true for the Colubridae (cobras).

Independence of the Spreading and Toxic Effect of Snake Venom

The injection of rattlesnake venom into the skin is followed almost immediately by a remarkably acute hemorrhagic necrosis. It might be argued that such a substance, by destroying all of the physiologic barriers to the spread of fluids in the tissues, would permit a spreading of any associated fluid or particulate matter. That this is not the case is shown by the following experiment in which the toxicity of the venom was markedly reduced by heating. The spreading factor from other sources has been shown to be comparatively thermostable (9).

Experiment.—4 samples of rattlesnake venom diluted 1:50 were heated for 25 minutes at 55°, 65°, and 95°, and for 5 minutes at 100° respectively. After eliminating the coagulated material by centrifugation progressive dilutions up to 1:1000 were prepared from each sample and 0.50 cc. of each dilution was mixed with 0.25 cc. of India ink or 0.25 cc. of saline. The ink mixtures were injected intradermally into one side and the saline mixtures into the other side of each of 4 rabbits. The results are given in Table II. This table gives also the average of 6 tests performed with unheated venom on separate rabbits for comparison.

The experiment shows that the venom heated at 65–100° has been largely deprived of its toxicity, but still retains the capacity to increase the permeability of the dermis.

The following experiment was carried out to find whether heated venom will increase capillary permeability.

Experiment.—2 rabbits were each injected intradermally with 0.5 cc. of each of 3 samples of rattlesnake venom, diluted 1:100 and heated 25 minutes at 65°, 85°, and 100° respectively. 2 more rabbits were similarly injected with dilutions of the unheated venom ranging from 1:5000 to 1:100,000. As a control, 0.5 cc. of saline was also injected into each of the 4 rabbits. Immediately after the intradermal injections, each animal received an intravenous injection of 4 cc. of a 0.1 per cent solution of T. 1824.[2] The intradermally injected venom began to spread at once and the localization of the circulating dye in the affected skin began immediately. Practically no localization of the dye occurred at the site of the saline injections during this time.

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[2] A poorly diffusible azo dye which remains a long time in the blood stream. Manufactured now by Eastman Kodak Company under the name of Evans blue.

At the end of 24 hours the sites injected with the venom heated at 65°, 85°, and 100° appeared as dyed areas measuring 47, 25, and 17 sq. cm. respectively. The sites of the injection of the 1:5000, 1:10,000, 1:50,000, and 1:100,000 dilutions of the unheated venom showed large blue areas measuring 72, 58, 29, and 26 sq. cm. respectively. Mild hemorrhagic lesions were observed in the areas injected with venom heated at 65°, and in those injected with the unheated venom, diluted 1:5000 and 1:10,000.

<div align="center">TABLE II</div>

Discrimination of the Spreading Factor of Snake Venom from the Local Toxic Factors as Demonstrated by Heating the Venom from a Rattlesnake
(*Crotalus adamanteus*)
(*Results after 24 Hours*)

Degree and time of heating of the venom solution	Area of spread and severity of lesion in rabbit skin injected with 0.05 cc. venom + 0.25 cc. India ink or saline				Area of spread of 0.5 cc. saline + 0.25 cc. India ink (control)
	Dilutions of snake venom				
	1:50	1:100	1:500	1:1000	
	sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
100° for 5 min.	17.8 Slight erythema	11.5 Slight erythema	9.0 No lesion	11.0 No lesion	2.7
85° for 25 min.	50.0 Slight erythema	44.5 No lesion	9.7 No lesion	7.8 No lesion	6.7
65° for 25 min.		45.6 Mild	22.5 Very mild	20.0 Very mild	7.8
55° for 25 min. (average of 2 tests)		100.0 Very pronounced	45.0 Pronounced	30.0 Mod. pronounced	5.3
Unheated (average of 6 tests)	125.0 Extremely pronounced	95.0 Very pronounced	45.0 Pronounced	30.0 Mod. pronounced	6.3

The experiment shows that the venom rendered practically atoxic by heating or dilution still retains its ability to increase the permeability of the blood capillaries, and thus allows the rapid escape of a circulating dye into the tissue spaces of the injected site.

In order to eliminate the complicating factor of the vascular reactions (hemorrhage and edema) brought about by the toxin, injections of venom were made into excised skin. It is known that the spreading factor from other sources exerts its action in the skin of the dead rabbit (10).

Experiment.—2 rabbits were killed by air embolism.   Then one received in one side three 0.50 cc. injections of rattlesnake venom in dilutions of 1:50, 1:100, and 1:1000, mixed with 0.25 cc. of India ink.   The skin of the second rabbit was excised, nailed down to a board and injected as above.   As a control, the usual saline and India ink mixtures were injected.   The bleb formed by the venom mixtures disappeared as promptly as when injected into the living animals.

After 24 hours the areas of ink spread where the venom was injected were from 3 to 6 times larger than the control areas.   In the excised skin the difference was less pronounced although quite clear.

The experiment shows that when snake venom and India ink were injected into the skin of dead rabbits the mixture spread through the tissue. The areas of spread were smaller than those which obtain in the living animal, and naturally, in the skin of the dead animal none of the edema and hemorrhage usually produced by the venom was present.[4]

Intravenous injection of venom was used as another means of eliminating the local toxicity of the venom.   It has been previously shown (2, 5) that spreading factors from testicle and bacteria, when injected into the vascular system, bring about an increase of permeability of the whole skin.

Experiment.—Mixtures of 0.50 cc. of saline plus 0.25 cc. of India ink were injected intradermally into each of 3 rabbits. The familiar discoid, convex bleb about 4 sq. cm. was formed in every case. 2 of the animals were then injected intravenously with 0.02 gm. of dry rattlesnake venom dissolved in 1 cc. of saline, while the third rabbit was left as a control. The injection of venom was followed by immediate signs of respiratory disturbance and the animals died in about 40 to 50 minutes. 4 more intradermal injections of ink were made at intervals while they were still alive and up to 2 hours after their death, and the resultant spreads were measured 5 hours after death. In every case the spreading was observed to involve an area 3 or 4 times as large as the bleb formed in the control rabbit. No gross evidence of tissue damage was observed in these areas.

Thus the spread of an India ink suspension in the skin is enhanced when the rabbit is inoculated intravenously with snake venom.   The permeability of the skin has been obviously increased by the cir-

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[4] The fact that final spreads in the living animal are much larger than in the dead one suggests that liquid from the circulating blood aids the spreading. An action of the spreading factor on blood capillaries must be thought of in this connection.

culating venom. Not improbably the latter passes into skin recently injected with ink as not elsewhere.

Enhancement of Virus and Bacterial Infections by the Spreading Factor

We have previously shown (2, 5, 6) that under certain conditions, the spreading factor greatly enhances experimental infections in the skin. These experiments were repeated with rattlesnake venom.

Experiment.—Samples of 0.5 cc. of venom were mixed with 0.5 cc. of dilutions of vaccine virus or a suspension of *Staphylococcus aureus* or bacillus of mouse typhoid II. Each resulting mixture of infectious agent and venom was injected

TABLE III

Enhancement of Virus and Bacterial Lesions by the Injection with Rattlesnake Venom (Crotalus adamanteus) Deprived of Toxicity

0.50 cc. venom or saline injected along with 0.50 cc. of suspension of the infectious agent	Area of lesions produced by infectious agent injected along with venom or saline				
	Results after 8 days			Results after 2 days	
	Vaccine virus 1:1000	Vaccine virus 1:100	Vaccine virus 1:10	Staphylo-coccus	Mouse typhoid
	sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
Venom 1:50 heated at 100°C.	7.4	12.4	34.7	23.6	16.5
Saline solution (control)	7.5	12.9	21.7	18.0	8.4
Venom 1:50 heated at 75°C.	30.8	75.9	68.2	42.4	20.8
Saline solution (control)	19.3	42.0	34.0	18.0	8.4
Unheated venom that has lost its toxicity 1:1000				69.2	
Saline solution (control)				18.0	

intradermally on one side of a rabbit. As a control, the infectious agent alone was injected on the other side. In order to eliminate the disturbing toxic factor of the venom, it was heated at 75° for 25 minutes or at 100° for 5 minutes. We also used a 1:1000 dilution of venom which we knew had lost most of its toxicity by long standing in the ice box. The results are given in Table III.

Venom heated at 75°, as well as unheated venom that had lost its toxicity on standing, markedly enhanced both bacterial and virus infections. Venom heated at 100° also enhanced the infections but to a much lesser extent than the other 2 preparations. Therefore, from the point of view of its effects on infection the spreading factor from rattlesnake venom behaves like the spreading factor from testicle and from invasive bacteria.

Action of Antivenom Serum on the Spreading Power of Snake Venom

Experiments were undertaken in an attempt to reveal with anti-toxin the extent of the linkage between spreading and toxic factors in snake venom. The antigenicity of the spreading factor from testicle, and from invasive bacteria had previously been investigated (11, 12).

Experiment.—3 rabbits were immunized against rattlesnake venom by an intradermal injection of 1 cc. of it, diluted 1:50, repeated twice later at weekly intervals. These injections produced the familiar local hemorrhagic lesions which, however, healed promptly. 10 days after the last injection the sera of the rabbits was found to possess a high titre of precipitins against rattlesnake venom. They

TABLE IV

Partial Suppression of the Spreading Power and Local Toxicity of Rattlesnake Venom (Crotalus adamanteus) in the Immunized Rabbit

Dilutions of venom	Amount of venom injected	Venom plus 0.25 cc. India ink or 0.25 cc. saline. Area of spread and severity of lesion							
		Immune rabbit				Normal rabbit (control)			
		40 min.	2 hrs.	5 hrs.	24 hrs.	40 min.	2 hrs.	5 hrs.	24 hrs.
	cc.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.
1:1000	0.50	6.9	12.5	18.0	15.0	7.7	12.5	18.0	20.0
		Slight ery-thema	Very mild	Mod. pro-nounced	Mod. pro-nounced	Mild	Mod. pro-nounced	Mod. pro-nounced	Pro-nounced
1:100	0.50	6.3	13.2	16.1	21.8	8.8	16.4	25.0	57.0
		Very mild	Mod. pro-nounced	Mod. pro-nounced	Pro-nounced	Mod. pro-nounced	Pro-nounced	Pro-nounced	Very pro-nounced
1:10	0.25		12.5	23.1	39.2		13.5	36.5	64.0
			Pro-nounced	Pro-nounced	Pro-nounced		Very pro-nounced	Very pro-nounced	Very pro-nounced

were now injected intradermally with dilutions of this venom mixed with either India ink or saline. Care was taken to choose sites in the skin which had not been previously injected. At the same time 3 normal rabbits were injected with the same venom dilutions. The resulting spreads and the character of the lesions in both groups of animals were recorded. In order to simplify the presentation of data, results from one control and one experimental animal are shown in Table IV. The other two pairs gave entirely similar results.

Experiment.—Dilutions of rattlesnake venom at 1:100, 1:1000, and 1:10,000 were respectively added to an equal volume of either normal serum or serum from a rabbit partly immune to the venom. The mixtures were allowed to remain in contact for 6 hours at 37°C. and overnight in the ice box. In those mixtures containing immune serum flocculation occurred, whereas the control mixtures remained clear. 4 rabbits were injected intradermally on one side with 0.50 cc.

of each of the immune serum mixtures (previously shaken), together with either 0.25 cc. of India ink or saline, and on the opposite side with the normal serum mixtures plus India ink or saline. The results obtained in every case showed that the mixtures containing venom plus immune serum spread the ink much less and produced milder lesions than those containing normal serum. Details from tests on 2 of the rabbits are shown in Table V.

These two experiments show that the specific antiserum neutralizes both the toxic factor and the spreading factor in snake venom, and that this neutralization takes place *in vitro* as well as *in vivo*.

TABLE V

Partial Suppression of the Spreading Power and Local Toxicity of Snake Venom (Crotalus adamanteus) by the Specific Antitoxin
(Results after 24 Hours)

	Area of spread and lesions produced by 0.50 cc. of mixture of venom and sera plus 0.25 cc. of India ink or saline					
	Immune serum			Normal serum		
Dilutions of venom...	1:100	1:1000	1:10,000	1:100	1:1000	1:10,000
	sq. cm.	*sq. cm.*	*sq.cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
Rabbit 1	35.5 Pronounced	19.3 Mod. pronounced	11.0 Mild	68.4 Very pronounced	26.2 Pronounced	14.2 Mod. pronounced
Rabbit 2	29.8 Mod. pronounced	17.9 Mod. pronounced	9.1 Mild	62.0 Very pronounced	22.0 Mod. pronounced	12.5 Mod. pronounced

Studies on the Venom from Toads

In the light of the above observations it was of interest to know whether spreading factors are associated with other animal poisons. The secretions from the parotid and other skin glands of toads were next studied from this point of view.

Experiment.—The dried secretions of the skin glands of 6 species of toads were each extracted with saline in the proportion of 1:50. After centrifugation 0.5 cc. of each extract was injected intradermally into one rabbit, along with 0.25 cc. of India ink or 0.25 cc. of saline. Control mixtures were injected using saline instead of venom. The results obtained were as follows:

The poisons of 3 species, *Bufo bufo*, *B. viridis viridis*, and *B. arenarum*, were found to be entirely devoid of spreading factor. Local lesions were absent or

extremely mild. However, the general toxicity which these poisons are known to possess was shown in our tests, since each rabbit injected died within a few hours. The poisons from 3 other species, *Bufo formosus*, *B. alvarius*, and Ch'an Su, proved to have a moderate amount of spreading factor, the areas of skin over which the ink extended being 2 to 4 times the control area. 2 of the materials produced a moderate local skin reaction. The third, that of *B. alvarius*, gave rise to no evident lesion. The general toxicity proved less with these last 3 venoms, the rabbits recovering after some intoxication.

These experiments show that the venoms of toads contains very little spreading factor or none, despite the fact that they are extremely potent poisons for the circulatory and nervous systems.[5]

DISCUSSION

It seems apparent from the above experiments that the venom from many snakes contains a factor capable of increasing the permeability of connective tissue and blood capillaries. Of considerable interest is the finding that the venom from various species of snakes differs considerably in spreading power, and apparently does so independently of its toxicity. Thus the spreading power is great in the Viperidae (rattlesnake) family, and relatively scant in the Colubridae proteroglypha (cobra) family. These two groups are characterized by the local action, and the neurotropic action, respectively, of their venoms. The secretions of the skin glands of toads are very toxic, and yet are almost devoid of any spreading factor. They also have very little local effect upon the tissues. Taking the facts together it would appear that a relationship exists between local tissue action and spreading factor content. This relationship may be analogous to that which has been discussed in connection with bacterial agents (5, 6). A distinction was drawn between invasive bacteria, which

[5] Besides snakes and toads, insects were also studied as possible sources of spreading factor (1). Their whole bodies were ground and extracted and for this reason the results obtained deserve only brief mention. It was found that extracts of spiders, bees, wasps, and mosquitoes injected together with India ink into the rabbit skin brought about areas of spread several times larger than the control even when used at high dilutions. Spider extract was the most active in this respect. On the other hand extracts of non-poisonous insects as, e.g. crickets, grasshoppers, dragon-flies, etc., were inactive. These preliminary results are only suggestive of a possible association of the venom with a spreading factor in the poisons.

liberate spreading factor, thus aiding their spread through the tissues, and virulent bacteria, which are devoid of spreading factor and rely upon other means to maintain and augment their status as pathogens. The locally acting rattlesnake venom containing spreading factor may be likened in this regard to *Clostridium welchii*, and toad venom may be likened to *Clostridium tetani*.

The relationship between the spreading factor and the toxins of snake venoms is not yet understood. The experiments conducted with heated venom, and those performed upon the excised skin, indicate that the two factors can be dissociated. On the other hand the specific antiserum of animals immunized with venom inhibits or suppresses both. In this connection it is of interest that extracts of the cells of the poison gland of the rattlesnake contain very little toxin, or spreading factor, but that both are present in the secretion of the gland (13).

CONCLUSIONS

The venom of several species of poisonous snakes acts to spread India ink through the skin as do the spreading factors procurable from certain tissues and elaborated by invasive bacteria. The factor is most abundant in the venom of the Viperidae (rattlesnake) family and relatively scant in the venom of Colubridae proteroglypha (cobra) family, and it is absent from toad venom. Extracts of the supralabial glands of harmless snakes contain only negligible amounts of the factor.

Rattlesnake venom heated at 65° to 100° loses a large proportion of its toxicity but retains the ability to spread ink.

Rattlesnake venom that has lost its toxicity on standing or on heating markedly enhances the infection produced by bacterial or virus suspension in the rabbit skin.

Antivenine serum inactivates both the toxic and spreading factors of venom.

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MIXED MOLECULES OF HEMOCYANINS FROM TWO DIFFERENT SPECIES*

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The investigations of Eriksson-Quensel and Svedberg (1) have accurately defined the molecular weights and pH stability regions of the hemocyanins. They have shown that the hemocyanins from a large number of different species are dissociated into smaller molecules when the pH of the solution exceeds the critical limit on either side of the stability region. In all species studied it was found that the dissociated molecules had weights which were simple fractions of the molecular weights of the native hemocyanins, *e.g.* 1/2, 1/4, 1/8, 1/16, etc. They have also demonstrated that the dissociation of hemocyanins is in almost every instance largely a reversible phenomenon and that upon returning the solution to a pH within the stability region molecules of the original weight are reformed.

From a consideration of these facts it seemed reasonable to speculate as to what would occur if the hemocyanins from two different species were mixed; dissociated into fractions and subsequently reassociated so that molecules of the original size were reformed. Two possibilities were quite evident: (*a*) the fractions of the hemocyanin molecules of each of the two species could only reunite with their own kind and would therefore reform the two original components, (*b*) the two different fractions could unite with each other as well as with their own kind and would therefore form an uncertain amount of a mixed hemocyanin besides the two original components. If the attractive forces which act between the dissociated fractions of the hemocyanin of a single species were considerably greater than those

which act between the dissociated fractions of the hemocyanins of different species, it was to be assumed that only the original components would be formed. On the other hand, if the forces which act upon the various fractions were independent of the species derivation of these fractions, a mixed hemocyanin could also be formed.

This problem is of considerable general importance since it is known that there is a very marked specificity in certain reactions between proteins, for example immunological and enzymatic reactions.

It has recently been demonstrated that chemically dissimilar substances even though they may possess similar molecular weights can readily be separated and identified by means of the electrophoretic technique (2).[1] The method has been found to be particularly useful in the isolation and identification of single molecular species in mixtures and has been successfully used with such complicated mixtures as, for example, mammalian sera. Although the molecular weights of the hemocyanins of certain species, *e.g. Helix pomatia, Helix nemoralis, Littorina littorea*, etc., are of the same order of magnitude, about 6,680,000 (1), the electrophoretic mobilities of these proteins are quite different (3). This difference in mobility under the influence of an electrical field is a property by means of which various hemocyanins of identical molecular weight can be identified in a solution containing those from two or more species.

It was thought to be probable that in the case that mixed hemocyanin molecules were formed upon the reassociation of mixed and dissociated fractions of hemocyanins from two species, these substances would possess sufficiently different electrochemical properties as compared with those of the native single hemocyanins to permit of their recognition electrophoretically. A quantitative study of certain characteristics of the electrophoretic technique with various hemocyanins as test objects has been carried out recently in this laboratory (4). The improved electrophoresis apparatus described by Tiselius (5, 6) has been used throughout these investigations. With the aid of the information which has been acquired in this more general study it was considered possible to approach the problem presented

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[1] Landsteiner, Longsworth, and van der Scheer (*Science*, 1938, **88**, 83) have recently reported differences in the electrophoretic mobilities of both egg albumins and hemoglobins from different species.

by the dissociation and reassociation of mixed hemocyanins from different species.

Because of the close biological relationship between *Helix pomatia* and *Helix nemoralis* the initial experiments were made with the mixed hemocyanins from these two species. Both hemocyanins have molecular weights of about 6,680,000 and both are completely dissociated into fractions of 1/8th the initial weight, about 810,000 at pH 8.5 (1). At pH 3.8 both these hemocyanins are approximately 40 per cent (7) dissociated into fractions of 1/2 the original weight, about 3,300,000 (1). Additional experiments were also carried out with the mixed hemocyanins from *Helix pomatia* and *Littorina littorea* which are much more distantly related biologically. At pH 8.5 the latter is partially dissociated into fractions of 1/2 and 1/8 the weight of the original molecules.

It is the purpose of this paper to report the production of a number of mixed hemocyanins each of which contains fractions of dissociated hemocyanins from two different species.

EXPERIMENTAL

Hemocyanins.—Whole blood was withdrawn from *Helix pomatia*, *Helix nemoralis* and *Littorina littorea*.[2] The blood was filtered through paper and dialyzed through cellophane against phosphate buffer (which contained 0.025 M Na_2HPO_4 and 0.025 M NaH_2PO_4) at pH 6.85 and ionic strength, μ, = 0.1 at + 4°C. Dialysis was continued for a period sufficiently long to achieve constant electrophoretic mobility of the hemocyanins (4). Between experiments the hemocyanin solutions were frozen and stored at − 10°C.

When it was desired to dissociate the hemocyanins they were dialyzed against 0.05 M NaCl for 18 hours at + 4°C. in order to free them of buffer. The desired buffer was then added directly to the hemocyanin solution. When dissociation was desired the following buffers were added to the freshly dialyzed solutions; for dissociation at pH 8.5, 18.0 cc. of 0.2 M KH_2PO_4 and 32.0 cc. of 0.1 M NaB_4O_7, for dissociation at pH 3.8, 20.8 cc. of 0.1 N HCl and 19.2 cc. of 0.1 M Na citrate. After standing for 1 hour at 20°C. the solution was then redialyzed against repeated changes of the phosphate buffer described above.

Electrophoresis.—The improved electrophoresis apparatus described by Tiselius (5, 6) has been used. In most instances the small apparatus with a sample capacity of 2 cc. has been used, while in certain experiments the larger apparatus

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[2] We are indebted to Dr. G. Gustafson of the Kristineberg Zoological Station for a supply of *Littorina littorea*.

with a U tube capacity of 11 cc. has been utilized. The entire electrophoretic technique has been kept as constant as possible from one experiment to another. In every instance the hemocyanin solution has been dialyzed against the same phosphate buffer, described above, and as routine this has been used as the supernatant solution over the hemocyanin in the U tube of the electrophoresis apparatus. The pH of the buffer has been determined by means of the hydrogen electrode and has varied only between 6.84 and 6.86. Conductivity of the buffer has varied only between 2.77 and 2.82 $\times$ 10^{-3} mho. The potential gradient, F, has been kept between 8.58 and 8.74 V/cm. in the smaller apparatus and between 9.04 and 9.22 V/cm. in the larger apparatus. All experiments have been carried out with the apparatus in the thermostat at between 0.3 and 0.7°C. In all experiments the scale method of Lamm (8) has been used. Photographs of the moving boundaries have been taken as routine at intervals of 25 minutes and more frequently when desired. The positions of the scale lines have been determined in the usual way by means of a microcomparator. The displacement of the scale lines, Z, in μ, has been plotted against the position of the displaced lines. In the diagrams thus obtained Z is proportional to the concentration gradient dc/dx in the cell. Electrophoretic mobilities have been determined both by means of the Toepler *Schlieren* method and also by means of the multiple scale method diagrams. The mobilities as determined by these two methods agree within 1 per cent.

Single Native Hemocyanins

Solutions of native hemocyanins from *Helix pomatia*, *Helix nemoralis*, and *Littorina littorea*, which had been dialyzed against the phosphate buffer described above, were studied in the electrophoresis apparatus. In the case of *Helix pomatia* and *Helix nemoralis* it was found that if adequate precautions were taken in the initial withdrawal of the blood and if the dialyzed solutions of the hemocyanins were stored frozen at −10°C. between experiments, these two hemocyanins migrated as single components. The hemocyanin of *Littorina littorea*, however, showed evidence of two components which migrated with somewhat different mobilities. The faster or main component constituted approximately 75 per cent of the hemocyanin while the slower or minor component comprised the remaining 25 per cent.

Examples of the electrophoretic scale method diagrams from three typical experiments with the native hemocyanins from these three species are shown in Figs. 1, 2, and 3.

All three photographs were taken 50 minutes after the electrical circuit had been closed at a potential gradient, F, of 8.74 V/cm. The very different migration distances of these three boundaries in a constant time interval demonstrates

graphically the differences in their electrophoretic mobilities since the experimental conditions were constant in the three experiments.

Fig. 1 shows the scale line displacement, Z in μ, produced by the moving boundary of a 0.96 per cent solution of native hemocyanin from *Helix pomatia*. It will be observed that only one component is present. This component has an electrophoretic mobility of -3.58×10^{-5} cm.^{2}, volt.$^{-1}$, sec.$^{-1}$.

Fig. 2 shows the scale line displacement, Z in μ, produced by the moving boundary of a 0.82 per cent solution of native hemocyanin from *Helix nemoralis*. In this case also but one component is present. This component has an electrophoretic mobility of -5.52×10^{-5} cm.^{2}, volt.$^{-1}$, sec.$^{-1}$.

FIG. 1   FIG. 2

FIG. 1. Electrophoretic diagram, scale method, of *Helix pomatia* hemocyanin, native, 50 minutes after closing electrical circuit. $F = 8.74$ V/cm. Migration, 9.18 mm. Phosphate buffer, pH 6.86, ionic strength $= 0.1$. u $= -3.58 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.96 per cent hemocyanin.

FIG. 2. Electrophoretic diagram, scale method, of *Helix nemoralis* hemocyanin, native, 50 minutes after closing electrical circuit. $F = 8.74$ V/cm. Migration, 14.26 mm. Phosphate buffer, pH 6.84, ionic strength $= 0.1$. u $= -5.52 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.82 per cent hemocyanin.

Fig. 3 shows the scale line displacement, Z in μ, produced by the moving boundary of a 0.80 per cent solution of native hemocyanin from *Littorina littorea*. The greater portion of the curve results from the faster component but the slower component is also plainly visible. These two components have electrophoretic mobilities of -7.64 and -7.05×10^{-5} cm.^{2}, volt.$^{-1}$, sec.$^{-1}$ respectively.

Additional scale photographs in these and in numerous other experiments failed to show any evidence of any components other than those illustrated for these native hemocyanins in Figs. 1 to 3. In certain experiments these three native hemocyanins were run for a period sufficiently long to permit of maximum migration in the electrophoretic apparatus.

Single Dissociated and Reassociated Hemocyanins

The solutions of native hemocyanins from *Helix pomatia* and *Helix nemoralis* were treated in the manner described above and were brought to pH 8.5 by the addition to them of borate buffer. At this pH both of these hemocyanins dissociate into fractions 1/8 the weight of the native molecules (1). The solutions were then dialyzed against repeated changes of phosphate buffer at pH 6.85 in order to cause reassociation and the reformation of molecules of the original size.
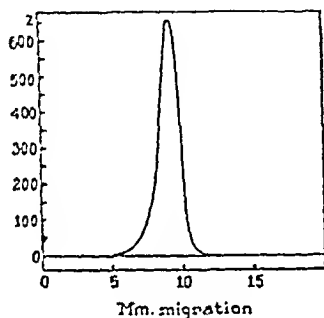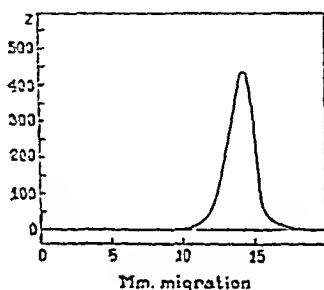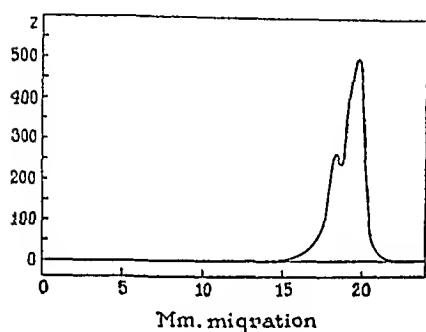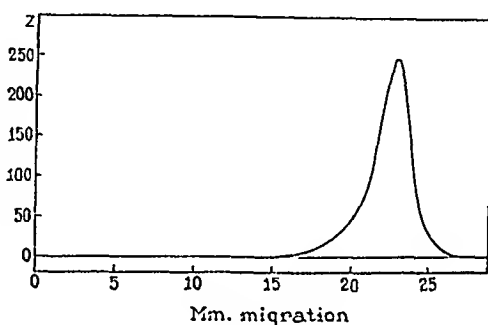
FIG. 3   FIG. 4

FIG. 3. Electrophoretic diagram, scale method, of *Littorina littorea* hemocyanin, native, 50 minutes after closing electrical circuit. $F = 8.74$ V/cm. Migration, 19.75 mm. Phosphate buffer, pH 6.85, ionic strength $= 0.1$. u $= -7.64 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.80 per cent hemocyanin.

FIG. 4. Electrophoretic diagram, scale method, of *Helix pomatia* hemocyanin, dissociated at pH 8.5 and reassociated at pH 6.8, 150 minutes after closing electrical circuit. $F = 9.22$ V/cm. Phosphate buffer, pH 6.84, ionic strength $= 0.1$. u $= -2.97 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.69 per cent hemocyanin.

Ultracentrifugal analysis of a similarly dissociated and reassociated solution of *Helix pomatia* hemocyanin indicated conclusively that molecules of the original weight had been reformed. In the case of both the native hemocyanin and the dissociated and reassociated hemocyanin there was only one homogeneous component in the ultracentrifugal diagrams. The former component had a sedimentation constant of 92.7 while the latter component had a sedimentation constant of 98.6×10^{-13}.

These dissociated and reassociated hemocyanin solutions were then studied in the electrophoresis apparatus. Examples of electro-

phoretic scale method diagrams from typical experiments with these solutions are shown in Figs. 4 and 5.

Fig. 4 shows the scale line displacement, Z in μ, caused by the moving boundary of a 0.69 per cent solution of *Helix pomatia* hemocyanin which had been dissociated at pH 8.5 and reassociated at pH 6.84. This photograph was taken 150 minutes after closing the electrical circuit at a potential gradient, F, of 9.22 V/cm. Only one component is present and this has an electrophoretic mobility of -2.97×10^{-5} cm.², volt⁻¹, sec.⁻¹. It will be noted that this mobility is significantly lower than that obtained with the native hemocyanin. A series of experiments with *Helix pomatia* hemocyanin, dissociated at pH 8.5 and reassociated at pH 6.8 have shown a quite constant decrease in the electrophoretic mobility, -3.08, as compared with that of the native hemocyanin, -3.53 (4).
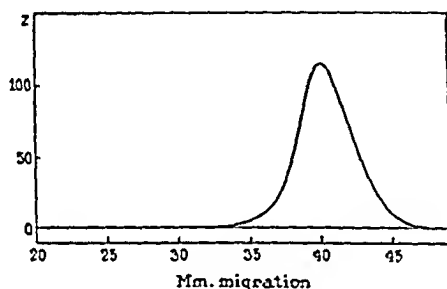
FIG. 5

FIG. 5. Electrophoretic diagram, scale method, of *Helix nemoralis* hemocyanin, dissociated at pH 8.5 and reassociated at pH 6.8, 150 minutes after closing electrical circuit. $F = 8.74\ V$/cm. Phosphate buffer, pH 6.85, ionic strength $= 0.1$. u $= -5.28 \times 10^{-6}$ cm.², volt⁻¹, sec.⁻¹. 0.42 per cent hemocyanin.

This has amounted to a 12 per cent reduction in the mobility under the stated experimental conditions.

Fig. 5 shows the scale line displacement, Z in μ, produced by the moving boundary of a 0.42 per cent solution of *Helix nemoralis* hemocyanin which had been dissociated at pH 8.5 and reassociated at pH 6.85. This photograph was taken 150 minutes after closing the electrical circuit at a potential gradient, F, of 8.74 V/cm. In this case also only a single component is present and this has an electrophoretic mobility of -5.28×10^{-5} cm.², volt⁻¹, sec.⁻¹. A decrease in the electrophoretic mobility after dissociation at pH 8.5 and reassociation at pH 6.8, similar to but less marked than that noted in the case of *Helix pomatia* hemocyanin, has also been found in the case of *Helix nemoralis* hemocyanin. This reduction in mobility does not exceed 5 per cent (4).

Although a large number of electrophoretic diagrams have been made with the solutions described above, in no case have additional components been observed after the dissociation and reassociation of these hemocyanins.

Because of the very small size of *Littorina littorea* it is very difficult to collect more than a small quantity of blood. In order to carry out all the other necessary experiments it was essential to conserve the small supply of this hemocyanin which was at hand. It was therefore not possible to determine its electrophoretic mobility after dissociation at pH 8.5 and reassociation at pH 6.8.

It will be observed that the boundaries in Figs. 4 and 5 appear somewhat wider at their bases than was the case with the boundaries of the same two native hemocyanins shown in Figs. 1 and 2. It will be recalled, however, that the photographs for Figs. 1 and 2 were taken at 50 minutes whereas those for Figs. 4 and 5 were taken at 150 minutes. Similar boundary spreading with increasing time occurs in the case of the native hemocyanins, as is well shown in Fig. 6, a photograph of native hemocyanin boundaries taken 125 minutes after closing the electrical circuit. The boundary-spreading phenomenon in electrophoresis and its significance have been discussed in another paper (4). It will suffice to state here that this phenomenon in no way interferes with the interpretation of the present experiments.

Mixed Native Hemocyanins

Solutions of native hemocyanins from *Helix pomatia* and *Helix nemoralis* were mixed so that the electrophoretic diagrams of the two single components could be studied simultaneously in the same solution. Solutions of native hemocyanins from *Helix pomatia* and *Littorina littorea* were similarly mixed for the same reason.

On the basis of the known differences between the electrophoretic mobilities of these three hemocyanins it was anticipated that in these solutions of mixed native hemocyanins it would be possible readily to separate and identify the individual components. This expectation was borne out by the experiments.

Examples of scale method electrophoretic diagrams from typical experiments with mixed native hemocyanins are shown in Figs. 6 and 9. In both instances the individual components are sharply defined and well separated from each other.

Fig. 6 shows the scale line displacement, Z in μ, produced by the moving boundaries of native hemocyanins from *Helix pomatia* and *Helix nemoralis* in a solution containing both hemocyanins. This photograph was taken 125 minutes after closing the electrical circuit at a potential gradient, F, of 8.58 V/cm. The electrophoretic mobility of the single *Helix pomatia* component, which has been designated H.P., is -3.39 while that of the *Helix nemoralis* component H.N. is -5.50×10^{-5} cm.^{2}, volt^{-1}, sec.$^{-1}$. This solution contained a total of 0.58 per cent hemocyanin of which 64 per cent was from *Helix pomatia* and 36 per cent was from *Helix nemoralis*.

Fig. 9 shows the scale line displacement, Z in μ, produced by the moving boundaries of native hemocyanins from *Helix pomatia* and *Littorina littorea* in a solution containing both hemocyanins. This photograph was taken 52 minutes after closing the electrical circuit at a potential gradient, F, of 8.64 V/cm. The solution
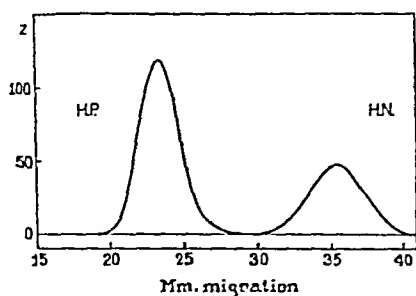
FIG. 6

FIG. 6. Electrophoretic diagram, scale method, of mixed *Helix pomatia* (H.P.) and *Helix nemoralis* (H.N.) hemocyanins, native. 125 minutes after closing the electrical circuit. $F = 8.58$ V/cm. Phosphate buffer, pH 6.86, ionic strength = 0.1. u (H.P.) = -3.39; u (H.N.) = -5.50×10^{-5} cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.37 per cent H.P. and 0.21 per cent H.N. hemocyanins.

contained a total of 0.93 per cent hemocyanin of which 56 per cent was from *Helix pomatia* and 44 per cent was from *Littorina littorea*. The electrophoretic mobility of the *Helix pomatia* hemocyanin, H.P., is -3.37 and those of the major and minor components of *Littorina littorea* hemocyanin, L.L., are -7.90 and -7.29×10^{-5} cm.^{2}, volt^{-1}, sec.$^{-1}$ respectively. In none of the experiments with these solutions of mixed native hemocyanins were any additional components observed. It will be noted that the mobilities found for the individual components in the mixed hemocyanin solutions correspond closely to those found for the single native hemocyanins.

Mixed Hemocyanins, Dissociated and Reassociated

The previous experiments reported in this paper had as their partial object the adequate control of the following experiments with

mixed hemocyanins which were dissociated and subsequently reassociated.

Solutions of mixed native hemocyanins from *Helix pomatia* and *Helix nemoralis* were treated as described above and were brought to pH 8.5 by the addition of borate buffer. At this pH both hemocyanins are completely dissociated into fractions of 1/8 the weight of the native molecules (1). The mixed hemocyanins were then reassociated at pH 6.85.

An example of the scale method electrophoretic diagrams obtained with these solutions is shown in Fig. 7. For comparison with the diagram obtained with the same mixed native hemocyanins it will be well to refer to Fig. 6. Both experiments were carried out under identical conditions and both photographs were taken 125 minutes after closing the electrical circuit.

Fig. 7 shows the scale line displacement, Z in μ, produced by the moving boundary of the hemocyanins which result from the dissociation at pH 8.5 and the subsequent reassociation at pH 6.85 of mixed hemocyanins from *Helix pomatia* and *Helix nemoralis*. The solution contained a total of 0.98 per cent hemocyanin of which 55 per cent was from *Helix pomatia* and 45 per cent was from *Helix nemoralis*. The potential gradient, F, was 8.74 V/cm. The electrophoretic mobility of the maximum peak of this curve is -3.97, whereas the mobilities of the fastest and slowest moving portions of the same curve are -5.25 and -2.50×10^{-5} cm.^{2}, volt $^{-1}$, sec.$^{-1}$ respectively.

A total of ten scale method electrophoretic diagrams were made at various boundary migration distances with this solution and in every instance only a single but a very rapidly spreading curve resulted. In none of the diagrams was there any separate component which had a mobility corresponding to that of the dissociated and reassociated hemocyanins of either *Helix pomatia* or *Helix nemoralis*.

Another solution of mixed native hemocyanins from *Helix pomatia* and *Helix nemoralis* after having been treated in the manner previously described was brought to pH 3.8 by the addition of HCl-Na citrate buffer. At this pH both hemocyanins partially dissociate into fractions of 1/2 the weight of the native molecules (1). The hemocyanins were then reassociated at pH 6.86.

An example of the scale method electrophoretic diagrams obtained with this solution is shown in Fig. 8. For comparison with the same native hemocyanins under similar experimental conditions refer to Fig. 6.

Fig. 8 shows the scale line displacement, Z in μ, produced by the moving boundaries of the mixed hemocyanins from *Helix pomatia* and *Helix nemoralis*, after these proteins had been dissociated at pH 3.8 and reassociated at pH 6.86. This photograph was taken 50 minutes after closing the electrical circuit at a potential gradient, F, of 8.66 V/cm. The solution contained a total of 0.41 per cent hemocyanin of which 55.5 per cent was *Helix pomatia*, 39.8 per cent was *Helix nemoralis*, and 4.8 per cent was a new component indicated by X in Fig. 8. The electrophoretic mobilities of these three components were -3.64, -5.55, and -4.35×10^{-5} cm.^{2}, volt^{-1} sec.$^{-1}$ respectively.
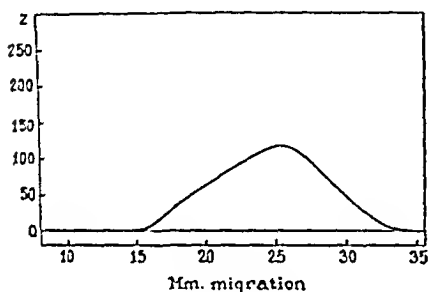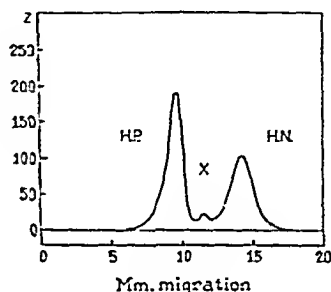
FIG. 7   FIG. 8

FIG. 7. Electrophoretic diagram, scale method, of mixed *Helix pomatia* and *Helix nemoralis* hemocyanins, dissociated at pH 8.5 and reassociated at pH 6.8. 125 minutes after closing electrical circuit. $F = 8.74$ V/cm. Phosphate buffer, pH 6.85, ionic strength $= 0.1$. u (max. Z) $= -3.97 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.98 per cent hemocyanin.

FIG. 8. Electrophoretic diagram, scale method, of mixed *Helix pomatia* (H.P.) and *Helix nemoralis* (H.N.) hemocyanins, dissociated at pH 3.8 and reassociated at pH 6.8. 50 minutes after closing electrical circuit. $F = 8.66$ V/cm. Phosphate buffer, pH 6.86, ionic strength $= 0.1$. u (H.P.) $= -3.64$; u (H.N.) $= -5.55$; and u (X) $= -4.35 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.23 per cent H.P. and 0.16 per cent H.N. hemocyanin.

A total of four separate scale method electrophoretic diagrams were made with this solution at various boundary migration distances and in each of the four diagrams the very small quantity of X component was present.

Solutions of mixed native hemocyanins from *Helix pomatia* and *Littorina littorea* were treated as described above and were then brought to pH 8.5 by the addition of borate buffer. At this pH *Helix pomatia* hemocyanin is completely dissociated into fractions 1/8 the

weight of the native molecules and *Littorina littorea* hemocyanin is partially dissociated into two fractions of 1/2 and 1/8 the weight respectively of the native molecules (1). The hemocyanins were reasociated at pH 6.86.

An example of the scale method electrophoretic diagrams obtained with solutions of this character is shown in Fig. 10. For comparison
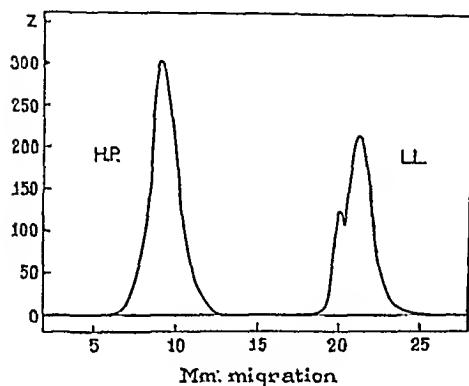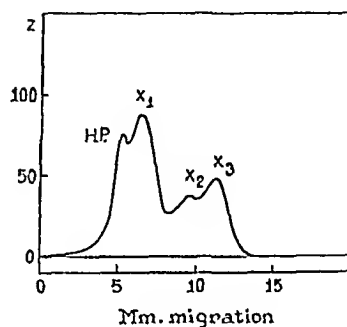
FIG. 9   FIG. 10

FIG. 9. Electrophoretic diagram, scale method, of mixed *Helix pomatia* (H.P.) and *Littorina littorea* (L.L.) hemocyanins, native. 52 minutes after closing electrical circuit. $F = 8.64$ V/cm. Phosphate buffer, pH 6.85, ionic strength $= 0.1$. u (H.P.) $= -3.37$; u (L.L. major) $= -7.90$; and u (L.L. minor) $= -7.29 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.52 per cent H.P. and 0.41 per cent L.L. hemocyanins.

FIG. 10. Electrophoretic diagram, scale method, of mixed *Helix pomatia* (H.P.) and *Littorina littorea* hemocyanins, dissociated at pH 8.5 and reassociated at pH 6.8. 40 minutes after closing electrical circuit. $F = 8.74$ V/cm. Phosphate buffer, pH 6.86, ionic strength $= 0.1$. u (H.P.) $= -2.93$; u $(X_1) = -3.57$; u $(X_2) = -4.90$; and u $(X_3) = -5.89 \times 10^{-5}$ cm.^{2}, volt^{-1}, sec.$^{-1}$. 0.49 per cent hemocyanin.

with the diagram obtained with a solution of the same mixed native hemocyanins refer to Fig. 9.

Fig. 10 shows the scale line displacement, Z in μ, produced by the moving boundaries of the hemocyanins which result from the dissociation at pH 8.5 and reassociation at pH 6.86 of mixed hemocyanins from *Helix pomatia* and *Littorina littorea*. The photograph was taken 40 minutes after closing the electrical circuit. The potential gradient, F, was 8.74 V/cm. The solution contained a total of 0.49 per cent hemocyanin of which 56 per cent and 44 per cent respectively were

the hemocyanins of *Helix pomatia* and *Littorina littorea* prior to dissociation. Four components are present in the diagram. These have been designated, in the order of increasing electrophoretic mobility as, H.P., X_1, X_2, X_3. These components had mobilities of -2.93, -3.57, -4.90, and -5.89×10^{-5} cm.^{2}, volt^{-1}, sec.$^{-1}$ respectively.

Nineteen separate scale method electrophoretic diagrams have been made at varying migration distances with solutions of mixed hemocyanins from *Helix pomatia* and *Littorina littorea,* in which the hemocyanins had been dissociated at pH 8.5 and reassociated at pH 6.8. In all of these diagrams there was evidence of the same four components, the electrophoretic mobilities of which remained practically constant in each diagram.

DISCUSSION

The dissociation at pH 8.5 and the subsequent reassociation at pH 6.8 of a mixture of hemocyanins from *Helix pomatia* and *Helix nemoralis* is followed by the reformation of molecules of the original size, just as is the case when either of these hemocyanins alone is similarly treated. The ultracentrifugal diagrams obtained with the mixture of hemocyanins which had been dissociated and reassociated showed the presence of but a single homogeneous component with a sedimentation constant of 94.7. However, the electrophoretic diagrams indicate the presence of a large number of different hemocyanin molecules with electrophoretic mobilities which range from that of the dissociated and reassociated hemocyanin of *Helix nemoralis* (e.g. -5.27), to somewhat less than that of the hemocyanin of *Helix pomatia* similarly treated (e.g. -3.08). These results indicate conclusively that a number of "mixed" hemocyanin molecules of the original size had been formed and that these contained fractions of both the original hemocyanins. It is known that both hemocyanins are completely dissociated at pH 8.5 into fractions about 1/8 the weight of the original molecules. If these various fractions are equally attracted one by another irrespective of the species from which they came and since upon reassociation molecules of the original weight are reformed it is feasible to calculate the minimum number of possible mixed hemocyanin molecules which could be produced in this system. It is also possible to calculate the probability that each of

these various mixed molecules will occur. If it can be assumed that the electrophoretic mobility of a mixed molecule can be calculated as the weighted average of the mobilities of its constituent parts it is possible to calculate the theoretical mobilities of each of the various mixed hemocyanin molecules which could be predicted.

Although the necessary assumptions have to be kept well in mind it has been of some interest to carry out the calculations indicated above.

TABLE I

Mixed Hemocyanins from Helix pomatia and Helix nemoralis, Dissociated at pH 8.5 and Reassociated at pH 6.8. Theoretically Possible Hemocyanin Association Products of Molecular Weight about 6,680,000

Number	Character	Constituents		Probability (P)	Theoretical mobility u × 10⁵
		Helix pomatia, 1/8 fraction Molecular weight about 810,000	*Helix nemoralis*, 1/8 fraction Molecular weight about 810,000		
1	Pure H.P.	8	0	0.0039	−3.08
2	Mixed H.P. + H.N.	7	1	0.0313	−3.36
3	" " "	6	2	0.1098	−3.63
4	" " "	5	3	0.2189	−3.90
5	" " "	4	4	0.2735	−4.17
6	" " "	3	5	0.2189	−4.45
7	" " "	2	6	0.1098	−4.72
8	" " "	1	7	0.0313	−5.00
9	Pure H.N.	0	8	0.0039	−5.27

The results are presented in Table I. It will be noted that in this dissociated and reassociated system nine different hemocyanin molecules are possible, as far as percentage composition is concerned. Two are the original pure hemocyanins from *Helix pomatia* and *Helix nemoralis*. The remaining seven are mixed hemocyanins which all contain fractions of hemocyanins from both species but in varying quantities. The probability that each of these nine possible hemocyanins will occur, as well as the theoretical electrophoretic mobility of each, is also given. It will be observed that all of the theoretical mobilities fall within the range covered by the electrophoretic diagrams obtained with a solution of mixed hemocyanins from *Helix pomatia*

and *Helix nemoralis*, which had been dissociated at pH 8.5 and subsequently reassociated.

In Fig. 11 the actual and the theoretical electrophoretic diagrams for this system have been superimposed. Curve I is the actual scale line displacement, Z, produced by the moving boundary of a solution of mixed hemocyanins dissociated at pH 8.5 and reassociated at pH 6.8, from *Helix pomatia* and *Helix nemoralis*. The displacements of the scale lines in μ have been plotted against the actual electrophoretic mobility of the components producing them. Curve II is obtained when the probabilities of the occurrence of the nine possible
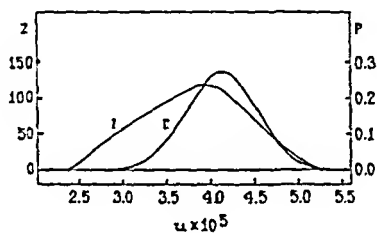
FIG. 11

FIG. 11. Curve I. Electrophoretic diagram, scale method, of mixed *Helix pomatia* and *Helix nemoralis* hemocyanins, dissociated at pH 8.5 and reassociated at pH 6.8. 125 minutes after closing circuit. $F = 8.74$ V/cm. The scale line displacement, Z in μ, has been plotted against the actual electrophoretic mobility, $(u \times 10^5)$ of the gradient producing it.

Curve II. The theoretical curve resulting from plotting the probability, P, of the occurrence of the nine theoretically possible hemocyanin association products in the above system against their theoretical electrophoretic mobilities.

hemocyanins in the above system are plotted against their theoretical electrophoretic mobilities. It will be observed that these two curves are not greatly dissimilar in the region of the faster components (*e.g.* $u \times 10^5 = -5.2$ to -4.0). The apparent discrepancy between the two curves in the region of the slower components (*e.g.* $u \times 10^5 = -4.0$ to -2.5) may be explained partly by the constant asymmetry of opposing halves of electrophoretic boundaries which has been described in detail in another paper (4).

From these considerations it seems possible that in the system just described there were at least nine different hemocyanin components

of which seven were mixed hemocyanins. Possibly there may have been many more than those indicated in Table I but it seems unlikely there were fewer.

The dissociation at pH 3.8 and reassociation at pH 6.8 of mixed hemocyanins from *Helix pomatia* and *Helix nemoralis* resulted in the reformation of the original molecules and probably also in the formation of a small amount of an additional mixed hemocyanin component which has been designated as *X*. At pH 3.8 both *Helix pomatia* and *Helix nemoralis* hemocyanins are partly dissociated into fractions 1/2 the weight of the native molecules (1). In the case of both proteins

TABLE II

Mixed Hemocyanins from Helix pomatia and Helix nemoralis, Dissociated at pH 3.8 and Reassociated at pH 6.8. Theoretically Possible Hemocyanin Association Products of Molecular Weight about 6,680,000

| Number | Character | Constituents | | Proba-bility (P) | Theo-retical mobility u × 10⁵ | Deter-termined mobility u × 10⁵ | Per cent of total hemo-cyanin deter-mined |
		Helix pomatia, 1/2 fraction Molecular weight about 3,300,000	*Helix nemoralis,* 1/2 fraction Molecular weight about 3,300,000				
1	Pure H.P.	2	0	0.250	-3.53	-3.64	54.9
2	Mixed H.P. + H.N.	1	1	0.500	-4.51	-4.35	4.8
3	Pure H.N.	0	2	0.250	-5.50	-5.55	40.3

the dissociation affects approximately 40 per cent of the molecules (7). If the assumptions which were made above are applied to this system it will be apparent that but one mixed component was to be expected. This component would contain equal amounts of the 1/2 fractions of the two dissociated hemocyanins. The fact that such a molecule would be definitely unsymmetrical in that it would be composed of chemically dissimilar halves might be a possible explanation for the fact that so small an amount of a new component was found in the electrophoretic diagrams. In Table II are shown the possible hemocyanin association products which could occur in this system. The actual and calculated mobilities as well as the determined and calculated quantities of each component are also given.

It seems possible therefore that the small quantity of a new component which was identified electrophoretically in this system repre-

sents a mixed hemocyanin composed of 1/2 fractions from *Helix pomatia* and 1/2 fractions from *Helix nemoralis*.

The dissociation at pH 8.5 and reassociation at pH 6.8 of mixed hemocyanins from *Helix pomatia* and *Littorina littorea* does not produce reformation of the original hemocyanins. Instead four distinct components are formed, which have been designated H.P., X_1, X_2, and X_3 in the order of their electrophoretic mobilities. At pH 8.5 the hemocyanin from *Helix pomatia* is entirely dissociated into fractions 1/8 the weight of the original molecules, while that from *Littorina littorea* is partly dissociated into fractions of 1/2 and 1/8 the weight of the native molecules. Biologically *Helix pomatia* and *Littorina littorea* are but distantly related and on this basis alone it seemed likely that difficulties would be encountered in the formation of mixed association products in solutions containing dissociated fractions of both hemocyanins. This difficulty has actually arisen for although three new components did appear in the electrophoretic diagrams it was found on ultracentrifugal analysis that the solution contained a number of components of different molecular weights. Furthermore the ultracentrifugal diagrams showed evidence of considerable inhomogeneity in these components. The various sedimentation constants ranged from about 90 to about 16. This indicates that the usually complete reassociation which was to be expected with the single hemocyanins in this system had in some manner been blocked. Since there has not been sufficient material available to permit of the electrophoretic isolation of each of the four components it has not been possible to determine their individual sedimentation constants. For this reason it is not feasible to attempt a calculation of the constituent parts of the possible mixed hemocyanins which may have been produced. The electrophoretic mobilities of the four components have been accurately determined and these are given in Table III.

The component designated H.P. is in all probability pure *Helix pomatia* hemocyanin since its mobility (-2.97) is practically identical with that of this hemocyanin when dissociated and reassociated under otherwise identical conditions (-3.08). The fractions X_1, X_2, and X_3 which have mobilities of -3.56, -4.87, and -5.85×10^{-5} cm.^{2}, volt^{-1}, sec.$^{-1}$ are probably products formed by the association of fractions from both the dissociated hemocyanins in the system. No

component which would correspond in mobility to either the maj
or minor component of *Littorina littorea* hemocyanin has been four
in any of the diagrams.   On the basis that dissociation and reassoci
tion may cause a 10 per cent reduction in the mobility of this hem
cyanin it has been calculated that in this system the major and min
components should have mobilities of -6.68 and -6.35×10
cm.^{2}, volt^{-1}, sec.$^{-1}$ respectively.

It should be stated that in none of the experiments with solutio
of mixed hemocyanins which had been dissociated and reassociate
was there any evidence that the various components were in a sta

TABLE III

*Mixed Hemocyanins from Helix pomatia and Littorina littorea, Dissociated at pH 8.
and Reassociated at pH 6.8.   Electrophoretic Mobilities of New Components*

Experiment No.	Electrophoretic diagrams Total number	Components			
		H.P.	X_1	X_2	X_3
		Mobility $u \times 10^5$			
1	4	-3.03	-3.51	-4.95	-5.91
2	9	-2.93	-3.57	-4.90	-5.89
3	6	-2.97	-3.62	-4.77	-5.75
Mean.......		-2.97	-3.56	-4.87	-5.85

of equilibrium.   In every instance the mobilities of the ascending and
descending boundaries of each component were similar.   The con-
centration of the various components did not vary systematically
with their position in respect to each other or with increasing migra-
tion distance.   It appears therefore that the various mixed hemocya-
nins which have been produced are probably as stable as the hemo-
cyanins from which they were formed.

The phenomena studied in the present paper may be of considerable
general interest for the understanding of the principles according to
which the huge protein molecules of the hemocyanin class are built up.
It is possible also that the environmental adaptations of certain viruses
and the carrier-shift of some enzymes are related phenomena.   Fur-
ther work with other mixed protein systems is needed, however, espe-

cially to find out more about the importance of biological relationship for the phenomenon of crossing.

It does not seem unlikely that the dissociation of hemocyanins and similar proteins into simple sub-multiples would indicate an ordered crystal-like structure of the individual particle or molecule. The cross-reactions studied here would then have their analogy in the formation of mixed crystals between substances of similar chemical structure.

SUMMARY

By means of the improved electrophoretic technique it has been possible to study the association products which are formed when mixed and dissociated hemocyanins from two different species are reassociated. Both the dissociation and the reassociation of the hemocyanins have been produced simply by altering the pH of the solutions. Scale method electrophoretic diagrams have been used throughout this investigation. This method has adequately demonstrated its advantages and has permitted exact calculations of the mobility, concentration, and homogeneity of components which were either present in too small an amount or were too nearly similar electrochemically to have been defined by other methods.

Evidence has been presented which indicates that a number of mixed hemocyanins have been produced. These mixed hemocyanin molecules contain fractions of the dissociated hemocyanins from two different species.

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THE MOLECULAR WEIGHT OF ANTIBODIES*†

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Antibodies as modified serum globulins are of special significance in investigations on the nature of the serum proteins. Many of the relationships of antibodies to the normal serum globulins, however, have not as yet been thoroughly studied. Immunological studies (1–3) have indicated differences between antibodies formed in the horse and in the rabbit and a subsequent study in the ultracentrifuge by Heidelberger and Pedersen (4) revealed a striking difference in sedimentation constant and hence probably in molecular weight. The therapeutic superiority of rabbit antipneumococcus sera over horse sera (5) also makes further knowledge of the relationships among antibodies of various species highly desirable. Among these properties, molecular weight and the effect of pH changes on antibody activity as well as on molecular size are of especial interest.

In this investigation Types I, III, and VIII antipneumococcus antibody were produced in the horse, cow, pig, monkey, man, and rabbit and were purified by the dissociation methods described in (6, 7). The sedimentation constants (s) were measured using the Svedberg ultracentrifuge (8), and the diffusion constants (D) determined in the Lamm diffusion cell (9). From these values the molecular weight may be calculated from the formula given by Svedberg:

$$M = \frac{RTs}{D(1 - V\rho)}$$

and it is also possible to calculate the frictional ratio f/f_0 which gives an indication of the deviation from the compact spherical shape.

Data on the effect of protein concentration on sedimentation and a correlation of antibody activity and the ultracentrifugal pH stability range are also included.

Methods

For the study of ultracentrifugal sedimentation as well as diffusion the modified Lamm scale method (10) was used. A brief summary of the ultracentrifuge method has already appeared (4).

Sedimentation runs were made at varying speeds from 45,000 to 70,000 R.P.M., lower speeds being used for the faster sedimenting molecules. All sedimentation constants were corrected for density and viscosity (8).

Diffusion runs were made, taking photographs of the diffusing boundary at varying time intervals (9). All values represent the average of three exposures for each diffusion run and are corrected for the viscosity of the solution.

The partial specific volume (V) of a horse antibody preparation was determined as 0.715, using a micro balance and a 2 ml. pycnometer with a 1.9 per cent solution. This value was assumed to hold for all species showing the antibody to be a heavy molecule. For the other species the value 0.745, previously found for serum globulin was assumed to be valid.

Except as noted all experiments were made in 0.15 M sodium chloride solution (physiological saline) thus making it possible to determine sedimentation constant, diffusion constant, and percentage of antibody on the same solution. Total nitrogen was determined by the micro Kjehldahl method and antibody analyses for precipitin or agglutinin were carried out by the absolute methods (11, 12). The factor 6.32 was used to transform nitrogen content to total protein.

RESULTS

The process of purification was followed in several cases in the ultracentrifuge by runs on extracts prepared by washing the specific precipitate or agglutinate at 37° with physiological saline (7) and also on the 15 per cent salt extract before and after dialysis against 0.15 M salt. Table I shows the results for a horse, a pig, and a monkey antiserum. The pig antiserum was very weak and was first precipitated according to Felton (13, 6). Data on the relative amounts of antibody and of the heavy protein component are also given. It will be noted that no evidence of dissociation or change in the heavy component occurs in the horse or pig preparations at any stage in the purification.

By use of the new analytical separation cell (14), it was possible to show that all of the antibody in a Type I antipneumococcus horse

serum 902 I was contained in the heavy serum component. After centrifugation until optical observation indicated that all the heavy molecules had just passed below the partition into the lower compartment, it was found that no antibody could be detected in the solution from the upper chamber with either Type I pneumococci or specific polysaccharide. This was also found to be the case for a purified pig antibody solution 198 F described in (7) and containing 72 per cent antibody. The sedimentation diagram showed two components of $s = 18.7$ and 6.7. The heavy component, as determined from the sedimentation diagram, was present to the extent of 77 per cent, in excellent agreement with the value obtained by analysis for antibody, especially since the amount of heavy component is usually greater than the antibody content, *i.e.* not all heavy component is antibody (Table I).

Table II is a summary of sedimentation and diffusion constants, molecular weights, and frictional ratios for the different antibodies purified from various species. Total protein concentration and per cent of antibody in the solution are given. Since some variation of sedimentation constants with concentration has been found, s and D were determined at the same concentration. All of the preparations gave quite uniform sedimentation peaks. In two preparations from monkey Type III antipneumococcus sera[1] a definite amount of a heavy component was present in the 15 per cent salt extract. On dialysis to 0.15 M salt, in one instance it seemed to have precipitated along with the insoluble material which usually forms, since it was no longer present in the 8-58 dialyzed solution (Table I). Owing to the limited amount of available material, no separation of the two was possible. The human antibody solution[2] was prepared from the serum of a convalescent Type I pneumonia patient containing 0.15 mg. of agglutinin N per ml.

Two preparations were made by separation of the globulin fraction in the Tiselius electrophoresis apparatus (15); one from normal human

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[1] Obtained in part through the courtesy of Dr. James D. Trask of the Department of Pediatrics, Yale University Medical School.

[2] Kindly furnished by Docent J. Waldenstrom, Upsala.

TABLE I

Sedimentation Constants of Horse, Pig, and Monkey Antibody at Various Stages in the Process of Purification

Species	Preparation No.	Description (7)	Concentration of solution	Antibody in solution (direct analysis)	Sedimentation constant $s_{20} \times 10^{13}$	Concentration of component in centrifuge	Appearance of components in sedimentation diagram
			per cent protein	*per cent*		*per cent protein*	
Horse Type I	902 I	Original serum	7.15	20.7			20 per cent heavy component in serum
	902 A	37° saline extract of washed specific precipitate	0.47	44	17.7	0.38	Single homogeneous component
	902 B	15 per cent salt extract at 37°	0.97	—	19.4*	0.95	" "
	902 B	Dialyzed against 0.15 M NaCl	1.35⎱ 0.58⎰	57	16.7 17.2		" "
	902 C	Ba(OH)₂, BaCl₂ treated residue	0.74	66	16.5 4.0		Definite breakdown of molecule; two equal major components; inhomogeneous material
						per cent of total protein	
Pig Type I	W	Original serum diluted 1:10	0.84	1.6	17.7†	9.7	Homogeneous heavy component in 1:10 dilution of serum
	W₁A	Felton solution diluted 3:5	0.89	10.3	17.8 6.8	42.8 57.2	Both components homogeneous Light component probably normal globulin

* Correction for viscosity and density more uncertain.

† s of heavy component in serum.

TABLE I—*Concluded*

Species	Preparation No.	Description (7)	Concentration of solution	Antibody in solution (direct analysis)	Sedimentation constant $s_{20} \times 10^{13}$	Concentration of component in centrifuge	Appearance of components in sedimentation diagram
			per cent protein	*per cent*		*per cent of total protein*	
Pig Type I	W_1B	Washed specific agglutinate; extract with 15 per cent salt	0.50	—	18.5		Single homogeneous component
	W_1B	Dialyzed to 0.15 M NaCl	0.56	84	17.4		" "
	W_1C	Ba(OH)$_2$, BaCl$_2$ treated residue	0.63	83	19.1 2.7		Considerable breakdown converted largely into small components and inhomogeneous
Monkey Type III	8-58	Original serum	9.25	6.6			
		15 per cent salt extract	—	—	17.0 6.8	17.3 82.7	Both components homogeneous
		Dialyzed to 0.15 M NaCl	0.14	>27‡	7.2		

‡ Probably somewhat in the inhibition zone because of too large an excess of polysaccharide.

serum and the other 431-5 from a highly potent anti-egg albumin serum (Table II).

It was observed that the sedimentation diagrams of two rabbit Ba(OH)$_2$, BaCl$_2$ dissociated antibody preparations 456$_2$ B and 453 C (6, 7) seemed to be no different from that of the 15 per cent salt dissociated antibody. Two other preparations, C and F, from another serum 475 showed small amounts of several heavier, more inhomogeneous components. The bulk of the antibody was unchanged, however.

Table III shows the variation of sedimentation and diffusion constants with concentration for a horse antibody preparation 902 E,

TABLE II

Physicochemical Constants of Various Preparations

Species	Method of purification	Preparation No.	Total concentration of solution	Proportion of antibody	$s_{20} \times 10^{13}$	$D_{20} \times 10^7$	Calculated molecular weight	f/f_0
			per cent protein	*per cent*				
Pig	15 per cent salt extract*	W₁B	0.58	84	18.0	1.64	930,000	2.0
Cow (7)	" "*	D₂	0.64	100	18.1	1.69	910,000	2.0
Horse	" "*	902 E	0.22	49	19.3	1.80	920,000	2.0
Monkey	" "*	8-58*†	0.31	40	6.7	4.06	157,000	1.5
	" "*		0.14	>27	7.2	3.50	196,000	1.6
Human being	" "*		0.39	44	7.4	3.60	195,000	1.5
	(γ globulin from electrophoresis)		(0.63)	Normal globulin	(7.1)	(3.84)	(177,000)	1.5
Rabbit (7)	15 per cent salt extract*	456₂ A	0.19	86	7.0	4.23	157,000	1.4
	Ba(OH)₂, BaCl₂*	456₂ B	1.08	94	6.3	3.77	156,000	1.6
	Ba(OH)₂, BaCl₂*	453 C	0.83	89	6.3	3.80‡	158,000	1.6
	γ globulin by electrophoresis	431-5§	0.53	76	6.5	3.75	165,000	1.6

* Of Pn-anti-Pn specific precipitate or agglutinated pneumococci.

† Values subject to a larger experimental error because of extremely small amount of material.

‡ Some slight drift in D for various exposures.   Mean value taken.

§ Anti-egg albumin.

TABLE III

Variation of Sedimentation and Diffusion Constant with Concentration of Solution for Horse Antibody 902 E

Concentration, *per cent protein*..........	0.22	0.45	0.90	1.35	1.80
$s_{20} \times 10^{13}$.....................	19.3	18.0	16.7	15.4	13.7
$D_{20} \times 10^7$.....................	1.80	1.83	1.84	1.63*	1.62

* 902 B.

made from the same bleeding of 902 I as preparation 902 B in Table I. For the study of concentration dependence and pH stability, the antibody from 200 ml. of serum was purified by salt dissociation of agglutinated pneumococci as in (7).   Fig. 1 is a graph of the variation of s with concentration.   The line is drawn through the points in Table III for 902 E and the other points were obtained with other horse, pig, and cow preparations at the concentrations indicated.   It will be noted that the diffusion constant remains fairly constant, but shows some drop in more concentrated solutions.

Fig. 2 shows the variation of the sedimentation constant with concentration for the smaller antibody molecule.   The data are taken from Table II.   It will be observed that the rabbit and monkey solutions give a smooth curve over the range of concentration studied. The human antibody preparation, as well as the normal human γ globulin, seems to have a significantly higher sedimentation constant.   Rabbit solution 456_2 B, however, run 5 months later at a concentration of 0.43 per cent seemed to have changed, giving a slightly higher value of $s = 7.0$.

Table IV is a tabulation of the effect of pH on the sedimentation constant and antibody activity for horse antibody solution 902 E. 1.5 ml. of 902 E 1.8 per cent solution in 0.15 M NaCl were mixed with 1.5 ml. of buffer solution made up in 0.15 M NaCl.   The solutions were ultracentrifuged 10 minutes after mixing and also after standing 48 or 72 hours in the ice box.   Antibody activity was determined by neutralizing and dialyzing against 0.15 M NaCl, after which samples were analyzed for agglutinin N and total N.

Antibody solutions 902 E and W1B were treated with 0.5 per cent phenol and let stand for 17 days.   After this time no change could be observed in the shape of the sedimentation diagram and values of s of 16.4 and 17.9 $\times$ 10^{-13} were obtained in 0.9 and 0.23 per cent solutions for 902 E and W1B respectively.

Table V is a summary of the horse antibody preparations showing more than one component.   In two instances antibody isolated from sera obtained early in the course of immunization contained only a single homogeneous component, while that from a bleeding after a longer period of immunization was markedly inhomogeneous.   The wide spreading of peaks and inhomogeneity makes the sedimentation

constants only approximate in these instances.   Rough estimates are also given for the total percentage of inhomogeneous material.

Antibody solution 902 K, prepared from the second bleeding of horse 902, and containing 26 per cent inhomogeneous material was run repeatedly in the separation cell, centrifuging the heavy component into the lower compartment until enough of the lighter components were available for analysis.   This solution containing the lighter inhomogeneous components showed immediate agglutination with Type I pneumococci and 19 per cent of the total protein was antibody.

<div align="center">DISCUSSION</div>

The present study is concerned chiefly with the ultracentrifugal behavior of antibody produced in various species of animals and purified by methods previously described (6, 7).   To detect any alteration in molecular size during the purification, solutions were run in the ultracentrifuge at each step in the procedure.   It will be noted (Table I) that all of the protein of low sedimentation constant can be washed away with saline at 0°, since solution 902 A, a saline extract at 37°, made after washing the specific precipitate at 0° until only traces of heat coagulable protein were present in the supernatant, contains only a single component of $s = 17.7 \times 10^{-13}$.   At no stage in the salt dissociation procedure was there any indication of alteration in molecular size of the material (Table I).   In the case of antibodies recovered from horse and pig sera the barium hydroxide dissociated material, however, showed definite breakdown in the ultracentrifuge although the antibody activity did not seem to be affected.   The rabbit antibody seemed more stable to the barium treatment.

Using the salt dissociation methods (6, 7), it has been observed that preparations from different antisera give antibody solutions which differ very markedly in antibody content, and also that a preliminary extraction of the washed specific precipitate with 0.9 per cent saline at 37° yields a lower grade antibody than a subsequent 15 per cent salt extraction.   Since in the original serum (902 I) the percentage of heavy component and of antibody is the same, and since the two salt extracts are indistinguishable in the ultracentrifuge, it is possible that some change affecting the antibody activity but not the molecular size might occur in purification.   Thus it has already been

observed (16) that purified antibody solutions contain more agglutinin than precipitin although they correspond in the original sera. Moreover, in the case of pig serum W, Table I, the amount of antibody in the original serum was much lower than the amount of heavy component, yet a solution containing 83 per cent antibody was finally obtained, indicating that the other non-specific heavy component did not interfere with the purification.

The data in Table II indicate quite definitely that the purified antibodies from the various animal species fall into two groups, those in which the antibody has about the same molecular weight as the ordinary serum globulins and those in which the antibody seems to be a much larger molecule. Goodner and Horsfall (3) have also observed a distribution of antibodies in two groups with respect to complement fixing ability and lipid composition, *i.e.*:

Complement fixing: Rabbit, rat; guinea pig, sheep.

Non-complement fixing: Horse, man, dog; mouse, cat, goat.

These properties do not, however, correlate well with the molecular weight data, since human antibody (a small molecule) does not fix complement and a preparation of cow antibody (a large molecule)[3] showed definite complement fixation.

Since both monkey preparations showed definite amounts of a heavy molecular species, it is possible that both types of antibody are formed in the monkey.

It is also of considerable interest to observe that the frictional ratio f/f_0 calculated in the last column of Table II is large for both groups of antibodies, indicating that they are either not compact or not spherical, since f/f_0 is equal to one only for a spherical unhydrated molecule. The heavy antibody molecule has one of the highest frictional ratios yet observed in a protein. It is therefore not surprising to find a marked dependence of sedimentation constant on concentration (Table III and Fig. 1). The cow and pig values obtained are in good agreement with the horse values. It seems, therefore, advisable to take the extrapolated value of sedimentation constant to zero protein concentration of 19.S. With 1.71 as an average diffusion constant for the three species and 0.715 for the partial specific volume,

[3] Kindly tested by Dr. A. J. Weil of Lederle Laboratories.

90,000 would seem the best value for the molecular weight of cow, horse, and pig antibody, using the Svedberg formula given above. In the horse antibody solutions there was no evidence of dissociation in dilute solutions as has been found for thyroglobulin (17).

Horse antibody 902 E showed very definite streaming double refraction in less than 0.1 per cent solution and measurements[4] indicated a
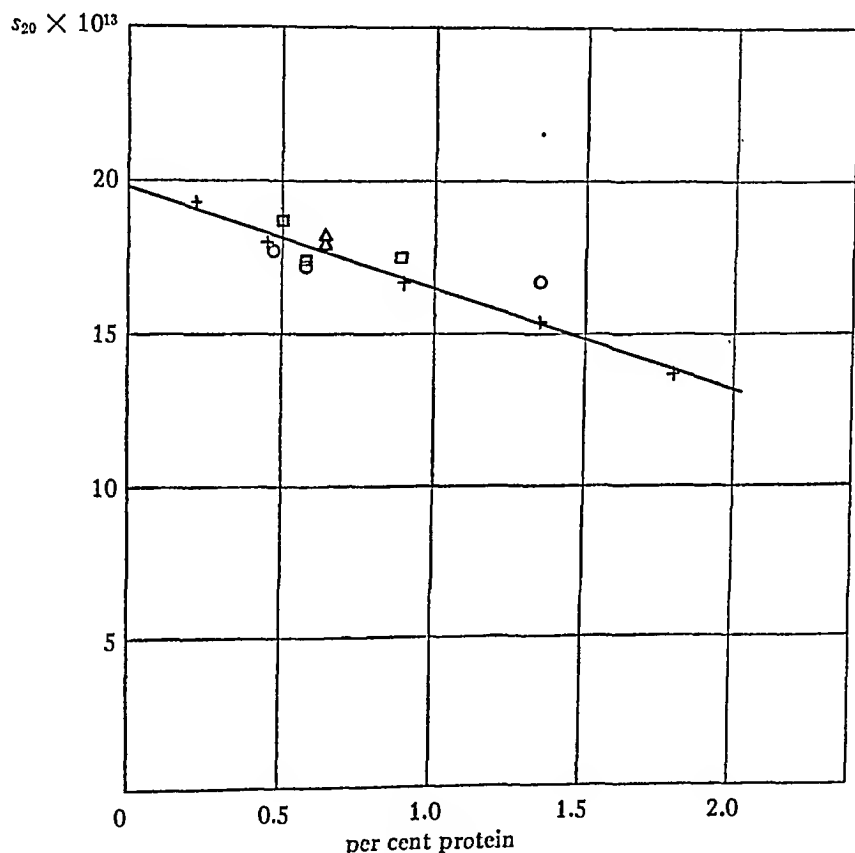
FIG. 1

+, horse 902 E.   O, horse 902 A and B.   □, pig.   △, cow.

length of not more than 100 mμ although the high dilution made accurate measurements very difficult. For a stiff cylindrical shaped molecule of molecular weight 990,000 and length 100 mμ, the ratio of the length to the diameter of the cylinder may be calculated as 25:1 as would be expected for a molecule with f/f_0 of 2.0.

[4] Kindly made by Dr. A. Snellman of this laboratory.

As shown in Table II and Fig. 2, the smaller antibody molecules also show definite variation of sedimentation constant with concentration. The diffusion constants also seem to show the same effect and the molecular weight in the rabbit and monkey preparations seems to be quite constant except for monkey 8-58 in which the weak solution available introduced a somewhat greater error. The best value for the molecular weight of rabbit and monkey antibody would be about 160,000, and since both the human antibody and the normal γ globulin both show a definitely higher sedimentation constant a probable average molecular weight would be about 185,000.
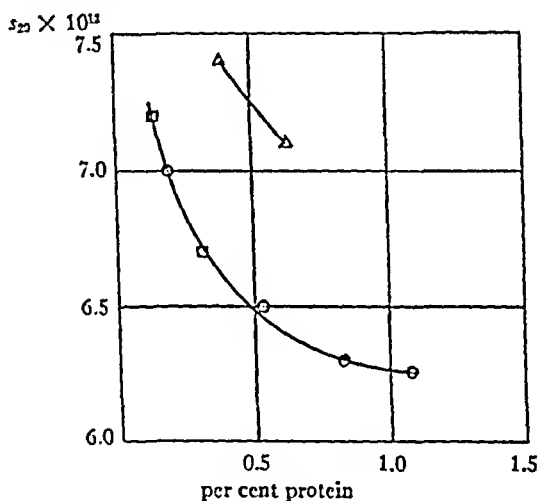
FIG. 2

○, rabbit.   □, monkey.   △, human being.

From the results in Table IV and Fig. 3, it will be seen that both antibody activity and molecular homogeneity are extremely stable to variation in pH, no appreciable change in activity occurring after 72 hours at pH 1.44 and after 48 hours at pH 10.9. The shape of the sedimentation diagram was unchanged between pH 3.4–10.9 although some association and dissociation seemed to take place below pH 4.88 and above pH 9.06 respectively. Some slight breakdown of the molecule was apparent at pH 1.44 with no loss of activity and complete loss of activity and destruction was found at pH 12.4. That this alkaline breakdown of the molecule may occur without loss of

TABLE IV

Effect of pH on Sedimentation Constant and Antibody Activity Horse Antibody 902 E. Concentration of Solutions in Centrifuge 0.9 Per Cent Protein

pH of solution	Total buffer in solution 0.15 M NaCl plus	Time of standing	Number of components in centrifuge	$s_{20} \times 10^{13}$ of main component	Antibody in solution	
					per cent	
1.44	0.015 M NaCl 0.05 M HCl	10 min. 72 hrs.	3 3	19.5 20.2	52*	Also small amounts of components $s = 6, 27$ Small amounts of components $s = 6, 30$
3.41	NaAc 0.01 M HAc 0.25 M	10 min. 48 hrs.	1 1	18.2 17.6		Single symmetrical peak " "
4.88	NaAc 0.025 M HAc 0.0125 M	10 min.	1	16.5		" "
7.65	No buffer	10 min.	1	16.7	49	" "
9.06	Na$_2$B$_4$O$_7$ 0.01 M	10 min.	1	16.3		" "
10.9	Na$_2$HPO$_4$ 0.025 M Na$_3$PO$_4$ 0.008 M	10 min. 48 hrs.	1 1	14.7 15.3	47†	" " " "
12.4	Na$_2$HPO$_4$ 0.05 M NaOH 0.10 M	10 min. 92 hrs.	2 2	6.7; 6.0 4.5	0	Two definite components complete breakdown Also some of a slightly lower component

* Antibody activity after 48 hours at pH 1.44. Neutralized solution showed main component $s = 18.4$ at 0.8 per cent concentration. Some slight breakdown of the molecule was still evident.

† Antibody activity after 72 hours in ice box, pH 10.9.

antibody activity may be seen from the data with the less drastic barium hydroxide dissociation methods (6, 7) as well as the barium hydroxide pig and horse preparations in Table I.

As noted by Heidelberger and Pedersen (4) not all preparations of horse antibody contained a single component. Even though the sera in the present experiments were free from preservative, many lots
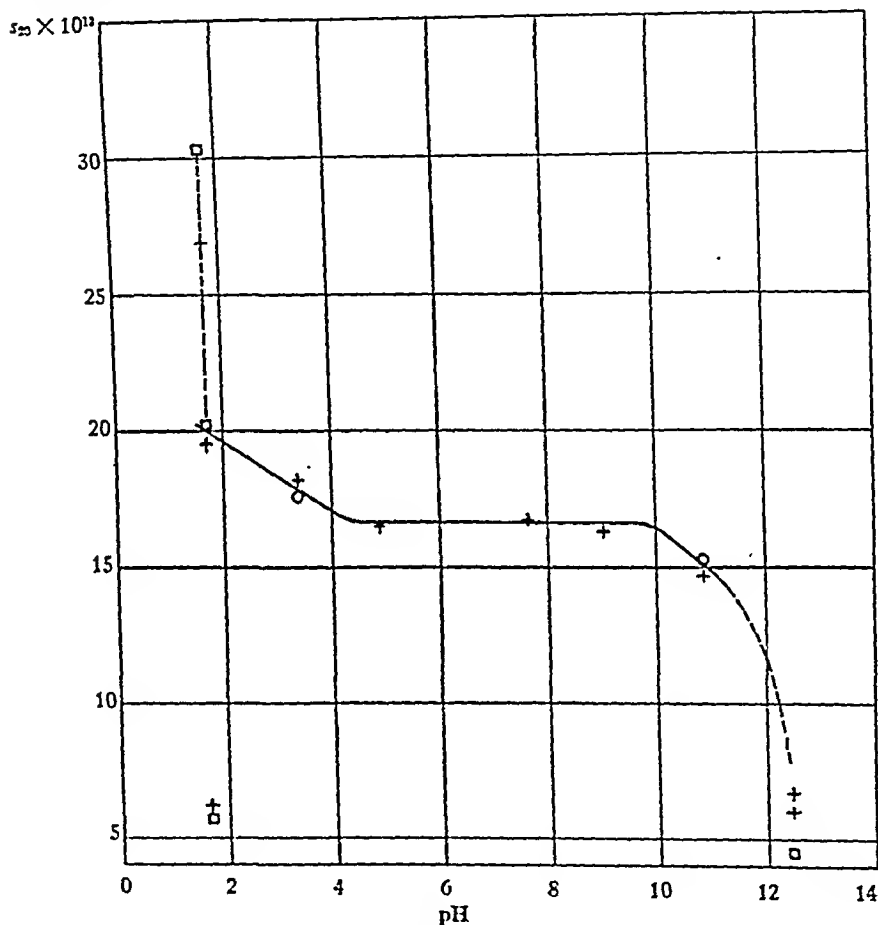
FIG. 3

+, 10 min. after mixing.
O, 48 hours "   "
□, 72   "   "   "

showed inhomogeneous components in amounts up to 65 per cent of the total protein present. That this can occur without seriously impairing the antibody activity is apparent from the high degree of

antibody content of solutions 792 EE, 9093 B, and 909 E.   A separation run in the analytical cell (14) also indicated that these degraded components possess antibody activity.   Since in two of the horses 902 and Sn,[5] an early bleeding yielded a serum from which antibody solutions containing only a single homogeneous component could be prepared, and since in each of these instances, bleedings after longer

TABLE V

Horse Antibody Solutions Containing More than One Component

Antibody solution No.	Concentration of solution	Antibody content	s of various components		Degraded inhomogeneous material	Description
	per cent protein	*per cent*			*per cent*	
792 DD	0.10	37	20.1	8.6	53	
792 EE	0.5	96	19.0	5.6	58	Also some of an intermediate component
9093 A	0.05	57	19.7	7	65	
9093 B in 15 per cent NaCl			16.2	7.4		Two components
B	0.46	86	17.6	10	33	
909 E	0.18	97	18.4	9	40	Also some intermediate components
902 I B	0.58	57	17.2	—	0	Homogeneous 1st bleeding
902 K in 15 per cent salt			18.0	7.6	27	
902 K	0.67	49	18.0	12.0; 10; 4	26	Second bleeding after 1 yr. of continued immunization
Sn I	0.68	61	18.2	—	0	Data from (4)
II A	0.18	6.5	19.2	10;8		Bleeding made several years later
B in 15 per cent salt			20.2	8		
B	0.63	30	18.6	17; 12; 7	22	

periods of immunization yielded solutions containing definite amounts of degraded material, it seems possible that the horse might develop means of breaking down the antibody into smaller aggregates without destruction of specific activity.   This might be an indication of a mechanism for removal of an otherwise minor protein component

[5] Stockholm horse used in (4).

from the blood stream when the animal is artificially stimulated to produce large quantities of such a component. It is interesting to note that both the cow and pig preparations studied were from animals under immunization for a comparatively short time and that only homogeneous antibody solutions were obtained.

The relationship of the heavy component of horse serum to a normal heavy component frequently present in small amount in sera is of considerable interest. A preparation of horse globulin[6] which was thought to contain heavy component showed two components, one having the sedimentation constant of normal globulin and the other heavier. Both components showed fairly symmetrical peaks in the sedimentation diagrams. To a sample of this solution, some homogeneous horse antibody was added and the mixture ultracentrifuged. The presence of three components $s = 19.0$, 10.9, and 6.9×10^{-13} indicates the non-identity of the normal heavy components in this preparation with the antibody.

SUMMARY

1. Highly purified preparations of homogeneous antibody can be made by the salt dissociation methods (6, 7) without any change in sedimentation due to the method of purification.

2. Antibodies prepared from sera of various animal species fall into two groups as regards molecular weight; in one group cow, horse, and pig, a heavy molecule of molecular weight 990,000 is formed; in human being, rabbit, and monkey, the molecular size is that of the normal γ serum globulin. Both types of antibody molecules are either not compact or not spherical since the frictional ratios f/f_0 are 2.0 and 1.5 respectively.

3. Horse antibody shows an unchanged activity and sedimentation diagram between pH 3.44–9.06, although there is some aggregation at the more acid and some dissociation at the more alkaline pH. At pH 1.44 the antibody activity is unchanged but some breakdown of the molecule takes place. At pH 12.4 activity is destroyed and the molecule is completely broken down.

4. Some horse antibody preparations show evidence of breakdown

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[6] Kindly supplied by Dr. F. E. Kendall.

of the antibody into inhomogeneous material on continued immunization over a long period.

We wish to thank Professor The Svedberg for his interest and for providing facilities for this work and Dr. Michael Heidelberger for supplying sera and making several of the antibody preparations especially for this purpose and for the interest taken in this work. We are also indebted to Dr. Kai O. Pedersen for many suggestions and continued guidance.

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AN ELECTROPHORETIC STUDY OF IMMUNE SERA AND PURIFIED ANTIBODY PREPARATIONS*†

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Studies on the electrophoresis of serum have shown that besides the albumin normal sera possess three separate globulin components differing in mobility and designated as α, β, and γ (1). The relationship of antibodies to these components is of considerable importance. Differences in the antibody globulin formed in various animal species have already been found by ultracentrifugal studies (2, 3) and in electrophoresis (4) as well as by immunological means (5). It has also been shown (1) that in an antiserum to crystalline egg albumin, the antibody migrated with the slowest (γ) component and could be isolated in one of the cells of the apparatus and analyzed for antibody content. The work reported in this communication is an attempt to study and compare the electrochemical properties of the antibody in the original sera with the purified antibody preparations described in (6) and to measure the isoelectric points of these purified preparations. It is also of importance to make use of and correlate the results obtained using both the mutually independent electrophoretic and ultracentrifugal methods since molecular weight homogeneity does not necessarily mean electrochemical homogeneity and vice versa. Since the methods previously described for determining the concentration of the various components are not sufficiently precise, especially in systems of several components, the Lamm scale method (7) has been used with the electrophoresis apparatus (8) and has provided a quantitative method of following changes in the various serum components on removal of antibody.

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Methods

The new Tiselius electrophoresis apparatus (8), in which sections of the U tube may be moved with respect to one another and cut off the column of solution into four parts to permit isolation of various components, was used.   The apparatus is designed to carry strong currents, which makes possible adequate resolution of the components of serum even in the presence of large amounts of salt.   Two methods of observation of the migrating boundaries were applied, Toepler's *Schlieren* method (9) and the Lamm scale method (7), both depending on refractive index changes in the solution due to the migrating boundary.   The former of these in which each component appears as a black band in the U tube image in the focus of the camera, provides a convenient way of following the experiment by direct visual observation.   In the latter method an equidistant scale placed behind the U tube is photographed at intervals through the solution.   These photographs are compared microscopically with those from a reference scale photographed before the current is started.   The displacements of the scale lines, Z, from their positions on the reference scale are plotted as ordinates against the corresponding positions in the U tube cell as abscissae yielding a curve in which each electrochemically distinct component in the solution will have its own peak (Fig. 1) provided the differences in mobility are sufficiently large and the experiment run long enough to produce adequate separation.   Since $Z = k \dfrac{dc}{dx}$, where k depends on the refractive index increment of the migrating substance and on known apparatus constants, it is evident that integration of the entire individual curve corresponding to a single peak will give the concentration, c, of the substance having the mobility of that peak.   One may thus obtain from the same curve the mobility and concentration of the electrochemically different proteins in the solution.   A micro modification of the apparatus in which 2 ml. of solution could be used was found convenient for the isoelectric point measurements on the purified antibody preparations.

In practice, however, since only data on the relative concentrations of the various components were desired and since nitrogen content and the area under each peak were related to protein concentration, it was not necessary to calculate absolute protein concentration in each case.   A base line for area measurements was determined by comparing the reference scale and photograph in the adjacent cells where no boundaries appeared.

It was also found convenient to compensate the boundary out from under the glass plates by a clockwork mechanism lowering an ebonite rod into the solution (8) before starting the current and then measuring the distance moved by an eyepiece scale fixed to the camera, using the *Schlieren* method, and in this manner to calculate the mobility.

All experiments were conducted in buffer solutions containing 0.15 M NaCl + 0.02 M total phosphates at the desired pH.   In the more acid range, the saline was buffered with acetate buffer in which the NaAc concentration was 0.02 M.   This

high salt concentration was found necessary since some of the horse and pig antibodies precipitated from solution if the usual 0.1 ionic strength buffers were employed. This procedure also enables the direct comparison of mobilities at different pH since the total ionic strength of the solution is but slightly changed by variation of the buffer and has the additional advantage of permitting the direct analysis of the solutions for antibody nitrogen by the absolute quantitative precipitin (10) and agglutinin (11) methods. The high salt concentration, however, does not permit the use of as high voltages as in 0.1 ionic strength buffer. A potential of 120 volts was usually employed giving a current of about 26 to 28 milliamperes and a potential gradient of about 3.4 volts per cm. with the ordinary apparatus. Higher voltages increased the risk of heat convection currents.

For the scale method experiments it was found advisable to use a 1:4 diluted serum to obtain optimum scale line displacements. In the case of unfractionated horse sera, however, it was not possible to determine concentration with the scale method since the curves did not come within a reasonable distance of the base line between components. This seemed also to be the case with the pig serum studied, but in rabbit and monkey antisera determination of concentration was quite satisfactory. These differences between horse and rabbit sera were most marked in higher concentrations and may perhaps indicate partial interaction or compound formation between the various globulin components of horse antisera (cf. Kendall, 12).

The usual procedure for studying the distribution of antibody in serum was to dilute the unabsorbed serum 1:4 and dialyze against buffer overnight. Another sample of the same serum was absorbed by addition of the proper amount of antigen and the combined supernatant and washings of the precipitate were diluted to the same volume as the unabsorbed serum and also dialyzed against buffer. In this manner the other components of the serum were present in unchanged concentration.

<p style="text-align:center">RESULTS</p>

Table I is a summary of the mobility data obtained for the antisera from different species in the saline phosphate buffer mixture at pH 7.72 ± 0.02. It will be observed that the mobility of all the components is less than in ordinary 0.1 ionic strength buffer (cf. 1). It can also be noted that in the horse antipneumococcus sera a new component migrating between β and γ was present. That this component disappeared on removal of the antibody (20.7 per cent of the total protein) can be seen from the *Schlieren* photograph (Fig. 2) of 902, before and after absorption of the antibody. No new component was observed in the other sera at the same pH. The mobility values, as measured for the slower components, would seem

to be subject to a somewhat greater experimental error in high salt concentration. In some cases, especially in concentrated solutions, the distance migrated did not correspond in each side of the U tube. This is a limitation of the method since not all of the components can be observed in more dilute solutions. As it was usually possible to get sharper bands on the positive side of the U tube, *i.e.* protein migrating into buffer, these values were used to calculate the mobilities listed in Table I.

TABLE I

Electrophoretic Mobilities of the Components of Sera from Various Animal Species at pH 7.72 ± 0.02 and in Buffer Containing 0.15 M NaCl + 0.02 M PO$_4^{\equiv}$ Temperature + 0.5°

Species	Serum	Anti-body	$u \times 10^5$ cm.^{2} sec.$^{-1}$ volt^{-1}				
			Albumin	α	β	Antibody	γ
		per cent					
Horse anti-pneumococcus I serum	{902 I undiluted	20.7	−5.5	−3.7	−3.0	−2.1	−0.9
	{902 II undiluted	21.8	−5.7	−4.0	−3.2	−2.2	−1.3
Normal horse			−5.7	−3.8	−3.1	—	−1.0
Pig antipneumococcus I serum	W undiluted	1.6*	−5.7	−3.5	−2.7	—	−1.1
Rabbit anti-egg albumin serum	431-5 1:4 diluted	36.4	−6.0	−3.6	−2.9	—	−1.1
Monkey antipneumococcus III serum	8-58 1:4 diluted	6.6	−5.2	−4.3	−3.0	—	−0.7

* This serum showed 9.7 per cent of a heavy component in the ultracentrifuge.

Table II is a summary of the results obtained by electrophoresis of rabbit and monkey antipneumococcus and anti-egg albumin sera before and after removal of the antibody. The type of curves obtained in the case of anti-egg albumin serum 431-5 is shown in Fig. 1 and the marked decrease in the γ component in the absorbed serum is evident. The absence of antibody in the α and β components was also directly established in the case of a rabbit antiserum to crystalline horse serum albumin by isolating the albumin and α and β components and demonstrating the absence of antibody with crystalline serum albumin.

Table III gives the mobility and isoelectric point measurements on

antibody preparations from different animal species. The details of the preparations are included in (3). All the preparations showed but one *Schlieren* band at each pH and the horse antibody preparation

TABLE II

Distribution of Antibody in Rabbit and Monkey Antisera

Species	Serum	Antibody by analysis		Total area under curve	Area γ component unabsorbed	Area γ component absorbed	Antibody area by difference	γ component in unabsorbed serum	Antibody in γ component	Antibody in original serum
		per cent		*sq.cm.*	*sq.cm.*	*sq.cm.*	*sq.cm.*	*per cent*	*per cent*	*per cent*
Rabbit anti-egg albumin	431-5	36.4	Positive side	229.4	119.8	34.4	85.4	52.2	71.2*	37.2
			Negative side	252.4	125.1	29.5	95.6	49.6	76.4*	37.9
Rabbit anti-egg albumin	446	12.3	Positive side	94.9	31.1	18.6	12.5	32.8	40.2	13.2
			Negative side	111.5	32.1	16.7	16.1	28.8	48.0	13.8
Rabbit antipneumococcus Type I	517	18.6	Negative side†‡	143.4	80.5	28.2	52.3	56.0	35.0	19.6
Monkey antipneumococcus Type III	8-58	6.6	Positive side	206.1	53.7	31.7	22.0	26.1	41.0	10.7
			Negative side	217.6	46.4	28.7	17.7	21.3	38.2	8.1

* Isolation of the component from this serum by electrophoresis yielded a solution containing 76 per cent antibody by analysis.

† Positive side results not used because of unsatisfactory reference scale; base line could not be accurately drawn.

‡ A different dilution was used for absorbed and unabsorbed sera; values given are corrected to the same dilution.

showed only one component by the scale method. In the case of the purified antibodies the distance moved in both the positive and negative sides of the U tube was practically the same and the values given are averages. The mobility pH curves are shown in Fig. 3. Insuffi-

cient monkey antibody was available for an isoelectric point deter-
mination. The mobility values obtained for the purified horse, pig,

TABLE III

Mobilities of Purified Antibodies at Various pH

$$u \times 10^5 \text{ cm.}^2 \text{ sec.}^{-1} \text{ volt}^{-1}$$

pH	Antipneumococcus					Anti-egg albumin
				Rabbit		
	Horse 902 E Salt dissociated	Cow D₂ Salt dissociated	Pig W₁B Salt dissociated	456₂ A Salt dissociated	456₂ B Barium dissociated	431-5 γ Isolated by electrophoresis
3.18	+2.21					
4.02	+1.12	+1.43	+2.15			
4.81	−0.63	0.00	+0.49	+1.04	+1.10	+0.95
5.86	−1.12	−0.98	−0.62	0.00	0.00	0.00
6.70	−1.34	−1.20	−1.02	−0.86	−0.60	−0.75
7.72	−1.70	−1.31	−1.30	−0.95	−0.85	−1.20
Isoelectric point pH	4.4	4.8	5.1	5.8(5)	5.8(5)	5.8(5)

TABLE IV

Electrophoresis of Purified Horse Antibody Showing Degraded Material in Centrifuge (Cf. 3) at pH 7.72

Preparation No.	Antibody	Number of components	u × 10⁵	Approximate concentration of degraded material in electrophoresis Per cent of total
	per cent			
9093 B	86	2	−1.6 / −0.3	46
902 K	49.4	2	−1.7 / −0.7(5)	22
Sn B	30	2	−1.8(5) / −0.9(5)	

and rabbit antibodies were the same as that observed in serum at the
same pH within experimental error.

As noted in Table I, pig W serum showed only four components at pH 7.72. From Table III and Fig. 3 it will be seen that at pH 7.72, the mobility of pig antibody is practically the same as that of γ component and hence they would not be separated at this pH. However, since the isoelectric point of pig antibody is much lower than that of γ component, a separation was possible by running the pig W serum at pH 5.86 (the isoelectric point of γ component) and five components were actually observed at this pH.

The production of inhomogeneous degraded components observed in the centrifuge (3) in several horse sera is paralleled in electrophoresis by the production of a new component of lower mobility than the heavy antibody. Table IV gives the mobility values and concentration of the components. 902 K is an antibody solution prepared from a bleeding from horse 902 a year after the bleeding from which 902 E (Table III) was made (*cf.* 3). This component seems to be present in a concentration about that shown in the ultracentrifuge.

DISCUSSION

The results now obtainable with the new electrophoresis technique (8) make it possible to obtain a more quantitative picture of the relationships of immune substances to the serum globulin and more detailed information about the electrochemical properties of immune sera and purified antibodies. Table I indicates that the mobilities of the various normal components in sera from several animal species are approximately the same. The immune horse serum, however, showed the presence of a new component, all of which seemed to be antibody, since it was absent on electrophoresis of the same serum after absorbtion of the antibody as shown in the photograph, Fig. 2. In the ultracentrifuge this serum also showed a heavy component which was the bearer of antibody activity (3). Various species of antibodies showing a heavy component in the ultracentrifuge, however, may not have a mobility sufficiently different from one of the other components at any definite pH to be observed as a distinct component in electrophoresis. Thus pig serum W containing 9.7 per cent of a heavy component in the ultracentrifuge showed only four components at pH 7.72. The purified antibody from this serum had a mobility so close to that of the γ component as to make effective

separation at this pH impossible. By selecting a pH at which the difference was more pronounced, the pig serum could be shown to contain five components.

The other group of sera in which the antibody was found to have the same molecular weight as the γ globulin fraction showed no new component. A comparison of the mobility pH curves (Fig. 3) shows no differences between rabbit antibody solutions prepared by salt dissociation (456_2 A), barium dissociation (456_2 B), or electrophoretic methods ($431\text{-}5\ \gamma$). The rabbit and monkey antisera, because of the wide separation of the peaks, were easily adapted to quantitative measurements. Table II and Fig. 1 give a comparison of the change in the electrophoresis scale diagram due to the removal of antibody. The values for percentage of antibody obtained by analysis for antibody nitrogen and total nitrogen (10, 11) (Table II, column 3) and those obtained by integration of the electrophoresis diagrams of the unabsorbed and absorbed sera (last column Table II) are well within experimental error and indicate that all of the antibody is contained in the γ globulin fraction. It will be seen that both sides of the U tube are in good agreement for percentage of antibody, but that the total area on the negative side is always greater than that on the positive side. This is perhaps due to the so called δ boundary (1) observed in most of these sera. The δ boundary is now thought to be a shift in total protein concentration due to a discontinuity in buffer concentration and conductivity formed near the starting position of the protein boundary in concentrated solutions (13). It has not been included in the curves. The agreement between the two sides in itself indicates that the occurrence of a δ boundary does not alter the relative concentration of the other components.

In those antisera in which the antibody occurs in the slowest migrating fraction, the electrophoresis method could be used to isolate fairly pure antibody. It is of great importance to select antisera in which a very high percentage of the γ component is antibody. On immunization there occurs an increase in total serum protein, as well as in total globulin and antibody (cf. for example 14); therefore determination of the amount of antibody nitrogen would not be a sufficient guide to purification by the electrophoresis method. The agreement between the percentage of antibody in the γ globulin
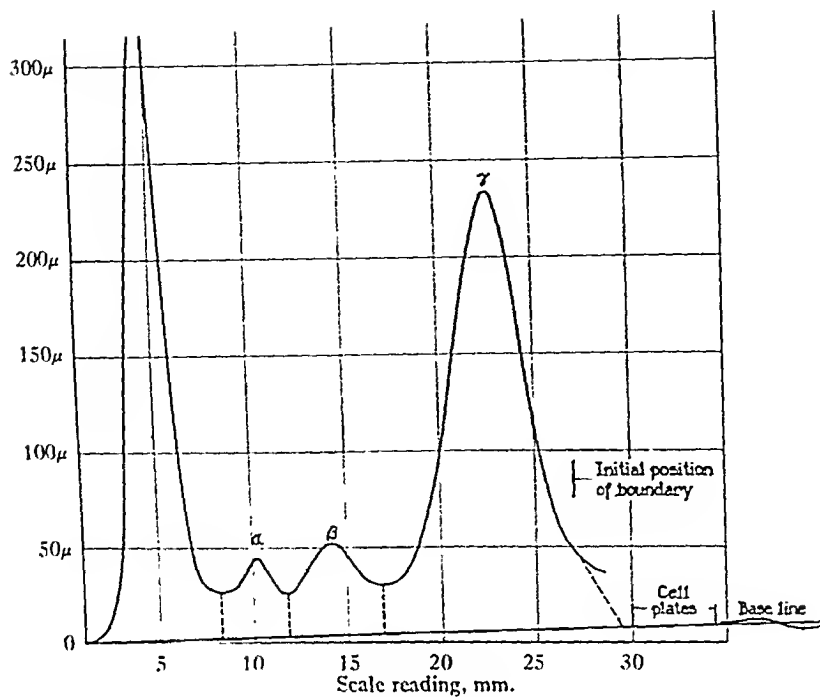
FIG. 1 a
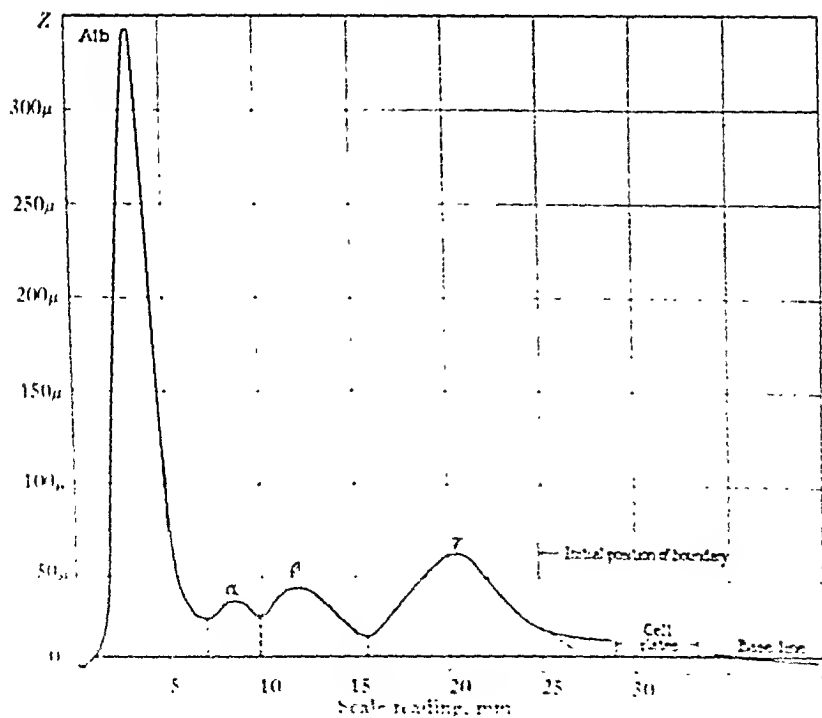
FIG. 1 b

FIG. 1. Electrophoresis scale diagram of anti-egg albumin rabbit serum 431-5. 1 b before and 1 after absorption of the antibody

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fraction of 431-5 (Table II) and that found by analysis after elec-
trophoretic purification suggests the advisability of a preliminary
run by the electrophoresis method as an indication of the suitability
of the method for purification of the antibody from a particular
antiserum.   A test bleeding from the animal could be taken and the
four times diluted serum run in the electrophoresis apparatus before
and after removal of the antibody.   From the scale photograph
(Fig. 1), the percentage of antibody in the γ component could be
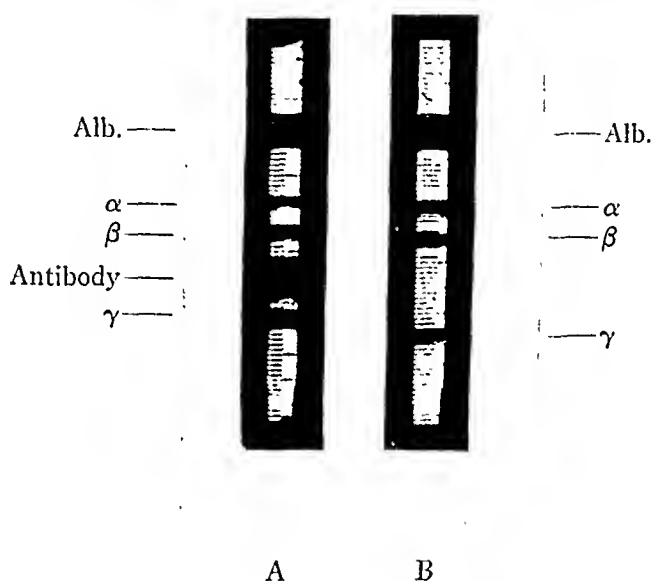
FIG. 2. Electrophoretic *Schlieren* photograph of unabsorbed (*a*) and ab-
sorbed (*b*) antipneumococcus horse serum.

calculated and the advisability of further immunization or of imme-
diate purification of the γ component decided.   For this preliminary
test, at most 5 ml. of serum would be required for the ordinary size
electrophoresis apparatus (8) but with a new cell of the same length
but of smaller cross-section area now being built, the required volume
of serum would be reduced to about 2 ml.   The method should be
most useful for obtaining purified antibodies to protein antigens since
the salt dissociation methods are not applicable.   In those cases in
which the antibody is not in the slowest component the method is too

laborious (such as horse antibody). The purified γ component is usually isolated in lower concentration than in the original serum due to the lack of absolute homogeneity of the globulin components (1). The advantage of the electrophoretic method is that it is a very mild method and permits isolation of proteins which retain their native state.

The isoelectric points of the different antibody preparations and the pH mobility curves are included in Table III and Fig. 3. The fact
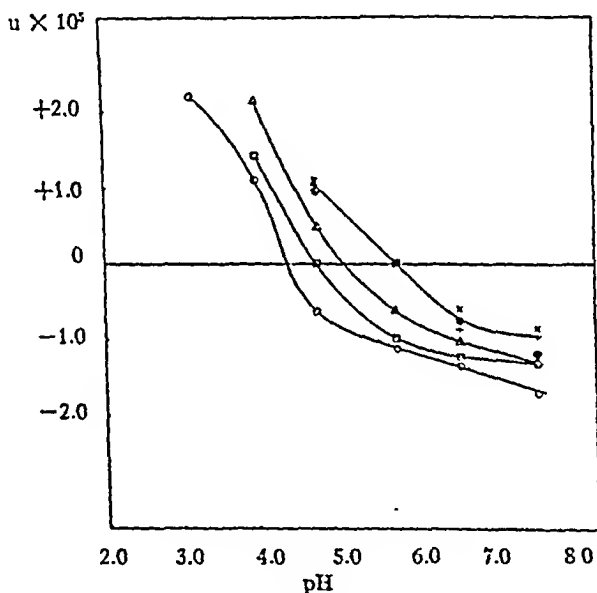
FIG. 3. Mobility of purified antibodies at different pH.
O, horse. □, cow. △, pig.
Rabbit 456₂ A + ; 456₂ B ×; 431-5 ⊖.

that these preparations showed only one *Schlieren* band does not indicate absolute homogeneity but merely that the purified preparations were no less uniform than any of the components observed in serum. The value for the isoelectric point of horse antibody is even somewhat lower than that given by Tiselius (4). Both these values are definitely lower than those given by Felton (15) and by Chow and Goebel (16). The methods of purification used by both of these

workers were non-specific precipitation methods.   As has been noted in the experimental section, the inability to integrate the scale diagrams in concentrated horse sera because of the poor separation of the globulin peaks may possibly be an indication of loose compound formation in these globulins and that the concentration methods of isoelectric precipitation used by these authors (15, 16) resulted in the precipitation of an addition compound of higher isoelectric point (*cf.* also Kendall, 12).

Additional evidence that the washed antigen-antibody precipitates (*cf.* 10, 11) are free from non-specific protein was obtained by dissolving a washed specific precipitate of egg albumin-anti-egg albumin in excess antigen and running in the electrophoresis apparatus.   Two components were observed, one of excess antigen and another of an antigen-antibody compound.   There was no component corresponding to ordinary γ globulin, indicating the absence of the corresponding non-specific γ component in the original washed precipitate.   A detailed study of the inhibition zone in electrophoresis has been completed.

The data concerning the degradation of horse antibody in the animal on continued immunization (3) indicate that it is also accompanied by a definite change in mobility as noted by the presence of two antibody components in a later bleeding from an animal which had previously shown one component (Table IV).   Thus 909₃ B showed two components in electrophoresis, both in appreciable concentration and a comparison of the original serum absorbed and unabsorbed showed that some of the slower γ component was removed on absorption of the antibody.   Similarly in horse antibody 902 E (Table III) prepared from an earlier bleeding, only one component was observed in electrophoresis and in the centrifuge (3), while in 902 K (Table IV), prepared in the same way from a bleeding taken after another year of immunization, degraded material was present in the centrifuge and a new component of lower mobility was present in electrophoresis. This is probably a manifestation of a process of degradation and removal of antibody protein in the horse after prolonged immunization.

SUMMARY

1. Antibody produced in the horse migrates as a new serum component between the β and γ components, whereas rabbit antibody

is electrophoretically identical with the γ globulin component of the serum.

2. In rabbit and monkey antisera the percentage of antibody in the serum and in the γ globulin fraction can be determined by integration of the electrophoresis diagrams of unabsorbed and absorbed sera. Antibody solutions of high purity can be obtained by electrophoretic isolation of the γ globulin of rabbit antisera in which the percentage of antibody to total γ globulin is high.

3. The isoelectric points of pig, cow, horse, and rabbit antibodies have been determined.

4. In horse sera prolonged immunization is accompanied by the formation of another antibody component of lower mobility.

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THE LYMPHATIC PATHWAY FROM THE NOSE AND PHARYNX

THE DISSEMINATION OF NASALLY INSTILLED VACCINIA VIRUS

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After noting that trypan blue and T-1824 (1), as also egg albumin and serum albumin (2), could be found in cervical lymph following nasal instillation, attention was directed to the fate of nasally instilled viruses. In this paper there are presented the results of experiments with vaccinia virus.

That vaccinia and other virus diseases can be transmitted by nasal infection has been shown by many workers. Calmette and Guérin (3) dusted dried and pulverized vaccinia virus into the nose of rabbits, and found that immunity developed. Gordon (4) showed that the nasal mucosa of rabbits is particularly susceptible to vaccinia. Rhoads (5), for 3 successive days, dropped a mixture (1.0 cc.) of equal parts of a 1:2,000 saline dilution of glycerolated neurovaccine brain virus and pooled rabbit immune serum into the noses of rabbits, and found that immunity was thereby conferred.

The virus of poliomyelitis presents features peculiarly its own, and is not considered in this paper. It has been responsible, perhaps more than any other virus, for the concept of neurotropic spread and efforts have been made to demonstrate such a mode of dissemination for several other viruses. Levaditi, Hornus, and Haber (6) showed that the intranasal instillation of herpetic virus gave rise to an encephalitis which was not prevented by interruption of the olfactory pathway. Levaditi and Haber (7), investigating the distribution of herpes virus during the incubation period, demonstrated its presence in the Gasserian ganglion, though not in the olfactory bulb, and suggested a spread along the fibres of the 5th cranial nerve. Sabin (8), working with the viruses of vesicular stomatitis, equine encephalomyelitis, and pseudorabies, was also of the opinion that viruses could spread along nervous pathways other than the olfactory, and that the virus

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of pseudorabies, for example, could extend either along the 5th cranial nerve, or along sympathetic and parasympathetic nerves.

Apart from nervous pathways, infective material introduced into the nasopharynx can spread in many directions. Some may be swallowed, some may enter the lungs. Mullin and Ryder (9) and Corper and Robin (10) showed that with rabbits in the recumbent position India ink suspensions dropped into the nose quickly reached the lungs, entering the terminal bronchioles and alveoli. In the present paper attention has been confined to the dissemination of nasally instilled vaccinia virus along the lymphatic pathway, though some virus may enter the blood stream directly after invasion of the mucosa.

TABLE I

Examination of Cervical Node and Lymph for Vaccinia Virus 0 to 9 Hours after Nasal Instillation

Experiment No.	Animal	Amount of virus suspension in each nostril	Time during which cervical lymph collected after dropping virus in nose	Virus present in	
				Superior deep cervical node	Cervical lymph
		cc.			
1	Rabbit	1.0	4 hrs. and 35 min.	None	None
2	Cat	1.0	4 hrs. and 30 min.	"	"
3	Monkey	1.0	4 hrs. and 50 min.	"	"
4	"	1.5	9 hrs.	"	"

Material and Methods

The experiments, which have been carried out on 4 monkeys (*Macaca mulatta*), 1 cat, and 16 rabbits, fall into two groups. In the first group (Table I) one or both cervical lymph ducts were cannulated under general anesthesia by intraperitoneal or intravenous nembutal. A suspension of vaccinia virus (1:10 dilution of infected egg membrane) was dropped into the nose, and the collection of lymph continued for periods ranging from 4.5 to 9.0 hours, after which the animal was killed. In the second group (Table II) 5 drops of virus suspension were introduced into each nostril under nembutal anesthesia, and the animals allowed to recover. Subsequently, at intervals ranging from 12 hours to 7 days, the animals were again anesthetised, and this time the cervical lymph ducts were cannulated. Lymph flowed spontaneously in the monkeys, but only after massage of the cervical ducts in the rabbits. The cervical lymph ducts were cannulated low down in the neck, immediately before their opening into the veins. Virus present in this lymph would, in the intact animal, have passed without further glandular interruption into the blood.

In addition to cervical lymph, in most experiments the following were examined:

(a) thoracic duct lymph, (b) blood, (c) superior deep cervical node (for its position in the cervical lymph pathway see (1)), and (d) scrapings of the nasal mucous membrane, or in Experiments 5, 6, 15, and 19 nasal washings.

Nasal washings were centrifuged and the supernatant fluid titrated. Nasal scrapings, obtained with a small curette inserted through the nostrils, were

TABLE II

Distribution of Vaccinia Virus 12 Hours to 7 Days after Nasal Instillation

Experiment No.	Animal	Time interval between dropping virus in nose and collection of lymph	Highest dilution of virus detectable in						
			Nasal mucosa*	Cervical node	Cervical lymph	Blood	Thoracic duct lymph	Popliteal node	Mesenteric node
5†	Monkey	4 days	+1:10	—	—	—	—		
6†	"	5 "	+1:10	—	+ Und.	—	—		
7	Rabbit	12 hrs.	+1:10	+1:10	+1:8	—	+1:4	—	
8	"	1 day	+1:160	—	+1:4	—	+1:4		
9	"	2 days	+1:160	+1:90	+1:16	—	+1:4		
10	"	2 "	+1:10	—	+1:16	—	+1:16	—	
11	"	3 "	+1:10	+1:12	+1:16	—	+1:4	+1:12	
12	"	3 "	+1:12	+1:80	+ Und.	—	—	—	
13‡	"	4 "	+1:100	+1:100	—		—	+1:40	
14	"	4 "	+1:160	+1:40	+1:4	—	—		
15†	"	5 "	+1:20	+1:50	+1:10	—	—		
16	"	5 "	+1:160	+1:40	+1:8	—	+1:4		
17	"	6 "	+1:160	+1:40	+1:16	+ Und.	+1:8		
18‡	"	6 "	+1:15,000	+1:40			+ Und.	—	—
19†	"	6 "	+1:1,000	+1:100	+1:4	—	+ Und.		
20‡	"	7 "	+1:40	+1:40	+1:8	—	+1:8		
21‡	"	7 "	+1:16,000	—	+1:4	+1:2	+1:4		—

Blank spaces indicate no examination. Und. = undiluted.

* In Experiments 9, 14, 16, and 17, the nasal mucosa was not titrated beyond 1:160. At this dilution the papules were large and well marked.

† Nasal washings.

‡ Fractionation experiments (see Table III).

weighed, ground, and centrifuged (2,000 R.P.M. for 20 minutes) to throw down sediment and most bacteria. Typical vaccinia lesions, uncomplicated by sepsis, were produced by the supernatant fluid. Since the curetting was done blindly, patches of less infected mucosa may have been obtained in some experiments as against more heavily infected areas in others. This may have been partly responsible for the wide fluctuations in the virus content of the mucosa.

In 6 experiments a popliteal lymph node was examined, in 2 the mesenteric. In 4 experiments cervical or thoracic duct lymph was centrifuged (2,000 R.P.M. for 15 minutes) and the sediment and supernatant fluid tested separately for the presence of virus. Lymph and blood were prevented from clotting by adding a few drops of a sterile solution of heparin. Lymph nodes were weighed before triturating and adding Tyrode's solution, so that the approximate dilution of virus was known. The amount of Tyrode's solution which had to be added for effective trituration and centrifuging, though kept at a minimum, may have diluted small amounts of virus beyond the concentration at which they could be detected.

Preparation and Detection of Virus

The vaccinia virus employed was obtained from crude fresh calf lymph supplied by the Massachusetts State Department of Public Health. This was ground with pyrex glass fragments in hormone broth (pH 7.6) and filtered at −40 mm. Hg through a Berkefeld V filter. The virus in the bacteria-free filtrate (titre 1:100,000) was propagated on the chorio-allantoic membrane of 12 day chick embryos and harvested after 48 to 72 hours. The membranes were stored at −20°C. until required, when they were triturated with pyrex glass fragments in Tyrode's solution. After centrifuging (500 R.P.M. for 7 minutes) the supernatant fluid contained virus which could usually be detected in a dilution of 1:200,000 (i.e., 1:2,000,000 of the original egg membrane). Virus was used only after passage through 5 to 20 egg generations. Examination for the presence of virus was by intradermal inoculation into a susceptible rabbit. Readings were made on the 3rd, 4th, and 5th days.

The following protocol of a typical experiment will illustrate the standard procedure.

Experiment 21.—Rabbit, weight 2.2 kg.
July 19, 1938. 2.0 cc. nembutal (5 per cent) intravenously. Rabbit placed on back. 5 drops of vaccinia virus in each nostril. Animal permitted to recover.
July 26, 1938. 8:50 a.m., nembutal 2.0 cc. (5 per cent) intravenously. *9:20,* trachea cannulated, stomach tube passed, 150 cc. saline introduced, esophagus ligated.
9:25, nembutal 0.5 cc. (5 per cent) intravenously. *9:50,* right cervical lymph duct cannulated, moderate lymph flow. *10:50,* left cervical lymph duct cannulated, flow light.
11:20, nembutal 0.75 cc. (5 per cent) intravenously. *11:30,* 50 cc. saline intravenously. *12:30 p.m.,* collection of cervical lymph (0.5 cc.) complete. Cannulae removed, and cervical ducts ligated.
2:00, 2.0 cc. thoracic duct lymph obtained.
2:15, blood sample taken from left jugular vein. Animal killed. *2:40,* left superior deep cervical node removed. *3:00,* scrapings made of nasal mucous membrane. *3:20,* abdomen opened, large mesenteric lymph node removed.

The tissues and fluids thus obtained were then titrated for virus content by intradermal inoculation into a test rabbit. Table III gives the details of this titration.

<div align="center">

TABLE III

Test Rabbit for Experiment 21

</div>

Tissue or fluid	Mode of preparation	Dilutions				
		Undi-luted	1:2	1:4	1:8	1:16
Lymphocytes from cervical lymph	Lymph centrifuged, super-natant fluid withdrawn and an equal amount of Tyrode's solution added to the sediment	++	++	+	−	−
Cervical lymph without lymphocytes	Supernatant fluid after centrifuging	−	−	−	−	−
Lymphocytes from thoracic duct lymph	Lymph centrifuged, super-natant fluid withdrawn and an equal amount of Tyrode's solution added to the sediment	++	++	+	−	−
Thoracic duct lymph without lympho-cytes	Supernatant fluid after centrifuging	−	−	−	−	−
Superior deep cervical lymph node	0.15 gm. plus 2.0 cc. Tyrode's solution	−	−	−	−	−
Mesenteric lymph node	0.7 gm. plus 2.1 cc. Tyrode's solution	−	−	−	−	−
Blood	Whole	+	+	−	−	−
		Undi-luted	1:10	1:100	1:1,000	1:10,000
Nasal mucosa scrapings	0.1 gm. plus 1.5 cc. Tyrode's solution	+++	+++	++	+	−

+++ large papule; ++ medium papule; + small papule.

Analysis of the Findings

Virus Found in Cervical Lymph only after 12 Hours.—In neither monkeys, cat, nor rabbits could virus be detected in the cervical lymph within 9 hours after having been placed in the nose (Table I). In the rabbits, with one exception, virus was found in the cervical lymph from 12 hours to 7 days after nasal instillation. The virus in these animals does not pass immediately through the mucosa, as

did dyes and proteins (1, 2). There seems rather to be a preliminary period during which the virus establishes itself in the nasal mucosa, which becomes reddened and hyperemic.

Virus Passes through Lymph Nodes.—The fact that virus is found almost constantly in cervical lymph (see Table II) after it has traversed a lymph node indicates that the nodes do not constitute an effective barrier to the passage of virus. Since there is no obvious difference in structure between cervical and other lymph nodes, it seems likely that this relationship holds good for all nodes.

Is the virus affected by its passage through the node? McMaster and Kidd (11) found that there was a time factor involved. Virus which had reached the cervical lymph nodes increased during the first 3 days and then began to diminish, having disappeared completely by the 11th day.

Virus Found in Thoracic Duct Lymph.—Virus is found almost as frequently in thoracic duct lymph as in cervical, though not usually in so great a concentration (Table II). The presence of virus in thoracic duct lymph may be due to some having been swallowed and invaded the gastro-intestinal mucosa.

Even when the quantitative end-points were the same, in so far as the accuracy of our titration permitted us to judge, there was a marked difference in the size of the lesions produced by cervical and thoracic duct lymph. The papules produced by the intradermal inoculation of the former were larger than those produced by the latter. Thus, in Experiment 16, taking the highest dilutions in which the two lymphs produced lesions, the cervical lymph papule was 7 mm. in diameter as against 4 mm. for thoracic duct lymph, and in Experiment 17 the corresponding diameters were 8 mm. and 4 mm., respectively.

Virus in Lymph Fixed by the Lymphocytes.—If a specimen of virus-containing lymph is centrifuged for 15 minutes at 2,000 R.P.M., virus will be found only in the sediment (Table IV). Centrifuging at this speed is insufficient to bring down free virus, as was readily confirmed by centrifuging ordinary virus suspensions. The virus therefore must have come down in association with the sediment, which consisted of a few red cells and large numbers of white cells, which smears showed to be almost 100 per cent lymphocytes. The work of Todd (12), Smith (13), and Douglas and Smith (14) would appear to exclude the red cell as a virus carrier.

One additional experiment (Table IV) seems to indicate that this fixation of virus by lymphocytes occurs *in vitro*, thus extending to the lymphocyte what has already been demonstrated by Douglas and Smith (14) and Sabin (15) for the blood leukocytes as a whole, by Rous, McMaster, and Hudack (16) for mixed cell suspensions from rabbit embryos, and by Beard and Rous (17) for isolated Kupffer cells.

Smith (13), analyzing the distribution of vaccinia virus in the blood, concluded that virus was never found in plasma or red cells, but was associated predominantly with the white cells, and in a much lesser

TABLE IV

Fractionation of Lymph*

Experiment No.	Source of lymph	Highest dilution of virus detectable in	
		Sediment	Supernatant fluid
13	Cervical duct	—	—
	Thoracic duct	—	—
18	Thoracic duct	+ Undiluted	—
19	Cervical duct	+ Undiluted	—
20	Cervical duct	+1:4	—
	Thoracic duct	+1:2	—
Normal thoracic duct lymph plus virus suspension†		+1:10	—

* Lymph centrifuged at 2,000 R.P.M. for 15 minutes. Supernatant fluid then removed and an equal volume of Tyrode's solution added to the sediment.

† The experiment is one in which 0.5 cc. of normal thoracic duct lymph and 1 drop of a 1:5 virus suspension were mixed, incubated at 37°C. for 2 hours (being shaken up at 15 minute intervals), and then centrifuged.

degree if at all with the platelets. "It is not known which type of white cell is of importance in this connection, or whether all types play a part. A few lymphocytes were usually found in the totally inactive suspensions of red blood corpuscles, but the number present was so small that they might have contained virus and yet have failed to produce any reaction." In the cervical and thoracic duct lymph of the rabbit the lymphocytes are for all practical purposes the only white cell to be reckoned with—other white cells are few and far between and the virus therefore must be fixed by the lymphocyte. Whether the virus is intracellular or adsorbed on the surface of the cell, virus and lymphocyte travel together.

Does fixation by the lymphocyte have any effect on the virus? Further experiments are in progress to determine this point. Several workers have studied mixtures of virus with other cells. Beard and Rous (17) found that vaccinia virus was fixed and largely neutralized by freshly obtained Kupffer cells. Rous, McMaster, and Hudack (16) showed that virus fixed by living cells (from cultures of rabbit embryo fragments) was protected against neutralization by antisera. On the other hand the cells themselves may have a destructive action on the virus. Fairbrother (18) found that a mixture of white cells and immune serum had a more potent neutralizing action than immune serum alone. The experiments of Douglas and Smith (14) suggest that immune plasma may have an opsonic effect on virus, since a mixture of immune plasma and normal blood leukocytes neutralize vaccinia virus to a greater degree than a mixture of normal plasma and washed leukocytes from an immune animal. Sabin (15) could not confirm their results.

The Lymph Node as a Centre for the Diffusion of Virus.—In respect to inert particles and bacteria (19, 20) the lymph node serves as a filter to lymph. With viruses there may be a fundamental difference. For since the virus becomes fixed by the lymphocyte, and since lymphocytes are constantly leaving the nodes in large numbers, it follows that unless the node becomes completely disorganized virus also must leave with the lymphocytes. This has been demonstrated in the present experiments for vaccinia virus; it has yet to be proved that the same mode of spread is valid for other virus diseases.

Significance of the Lymphocyte as a Virus Carrier.—That virus can be fixed by the lymphocyte may be significant for several reasons. (*a*) The virus may be protected in its passage through body fluids against any neutralizing principle which they may contain (16). (*b*) Since the lymphocyte can migrate through the walls of the capillaries, virus which has entered the blood stream can leave it in all parts of the body, and the finding of vaccinia virus in any given tissue becomes explicable by blood transmission. This does not exclude the possibility of other modes of spread, but may render them more difficult to prove. (*c*) One of the most striking pathological changes in so many virus diseases is the perivascular accumulation of lymphocytes. This is usually interpreted as reaction to the virus. May it

conceivably, at any rate in part, be cause rather than effect?   May it be that the accumulation of lymphocytes carrying virus is responsible for the first introduction of virus into the affected region?

In conclusion, we would like to record our indebtedness to Dr. Cecil K. Drinker and Dr. John F. Enders for advice and encouragement.

CONCLUSIONS

Vaccinia virus dropped into the nose of a susceptible animal does not appear in cervical lymph in less than 12 hours.   After 12 hours, and thereafter continuously for 7 days, a stream of virus is entering the blood through the cervical lymph ducts.   Virus passes through the lymph nodes.   Virus is fixed by the lymphocytes, and the significance of this is discussed.

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A PRECIPITINOGEN IN THE SERUM PRIOR TO THE ONSET OF ACUTE RHEUMATISM*

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The evolution of a rheumatic attack involves three distinct clinical phases (1, 2). The first is an acute respiratory infection, usually of not more than 3 days duration. The second is an afebrile, symptom-free period which may vary somewhat but commonly lasts 14 days. The third is the period of acute rheumatism, composed of one or more cycles of activity. Cycles consist of about 10 to 14 days of disease activity separated from each other by 7 to 10 days of remission. Of these three phases, the second appears to be crucial in the genesis of rheumatic activity. In an intensive study of this symptom-free period we have detected in the past only one abnormality, a diminution of serum complement (3).

Investigators in Jena (4), Wurzburg (5), Turin (6), Jerusalem (7) and New York (3), are agreed that during acute rheumatic fever serum complement may fluctuate from the normal to low levels, becoming steady at a normal level with recovery. With the exception of acute nephritis (8) and serum sickness,[1] a wide variety of other diseases show little or no variation in the complement level (9).

Whether the decrease in titratable complement observed during the course of rheumatic fever is a result of the rheumatic process or a factor in the pathogenesis of the disease cannot be decided *a priori*. However, the decrease which occurs in phase II, before the onset of acute rheumatism, is definitely not a result of rheumatic activity,

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[1] Unpublished data from this laboratory.

lending further support to the possibility that the phenomenon may be a factor in the pathogenesis of the rheumatic attack.

Decreases in serum complement are known to occur when there are present in the circulation both an antigen and its antibody, with the former in excess. This suggested that during phase II, when complement is diminished an excess of some antigen might be present. Should such an antigen occur, its antibody should then be present in excess shortly after the return of complement to normal, that is in the course of phase III. We have therefore looked for evidence of both antigen and antibody in the form of mutual precipitation between sera of phase III (presumptive antibody) and sera from phases II and I (presumptive antigen).

EXPERIMENTAL PROCEDURE

Selection of Patients.—Serial blood samples were taken at least once a week from rheumatic subjects recovering from hemolytic streptococcus pharyngitis, and from non-rheumatic controls. Single blood samples were taken from control patients with other febrile disease and from individuals in good health.

Preparation of Sera.—Sera were preserved with merthiolate and stored at 5°C. for periods up to 6 months. Before being tested, they were cleared by high speed centrifugation and, when necessary, by filtration through a Pasteur-Chamberland candle L2.

The Precipitin Test.—Chemically clean precipitin tubes and pipettes were used exclusively. Undiluted sera were tested against one another for mutual precipitation. 0.1 cc. of antibody serum was layered over 0.1 cc. of antigen serum. Three readings were recorded as follows, the third being the one reported throughout this paper: (*a*) Ring formation after 10 minutes at room temperature. (*b*) Precipitation after mixing and incubation for 2 hours in a 37° water bath. (*c*) Precipitation after refrigeration overnight at 5°C. The intensity of precipitation is expressed as in previous communications (10).

+++ large flakes.

++± large and small flakes.

++ small flakes or granules supernatant clear.

+± numerous flakes or granules appearing in cloudy whirl.

+ cloudy whirl.

0 clear.

− not tested.

Preliminary Observations

It was soon observed that sera taken at the height of an acute attack showed definite precipitation with sera taken within a week

before the onset of symptoms. Pairs of sera which reacted with each other were then tested separately against other sera from the same patient, as tabulated in the following sample protocol.

Phase	Date	Phase I	Phase II		Phase III (cycle I)		
		(May 10)	(May 18)	(May 22)	(May 24)	(May 28)	(June 1)
I	May 10		−	0	0	0	0
II	" 18	−		0	+	++	+
	" 22	0	0		−	++	−
III	" 24	0	+	−		−	−
	" 28	0	++	++	−		0
	June 1	0	+	−	−	0	

These findings showed that precipitation occurred only between phase II and phase III sera. In at least six other patients known positive phase II sera were tested against each other and known positive phase III sera were also tested in pairs. No reactions occurred between any of these combinations. This limitation of the reaction to the combination of phases II and III furnished a plan on which extensive investigations were based. Sera from phase II (and also, when available, from phase I) were tested as potential antigens against sera from phase III.

Precipitin Reactions in Acute Rheumatism

The first data to be discussed are from seventeen rheumatic subjects who developed rheumatic fever following hemolytic streptococcus pharyngitis during the winter and spring of 1938. Four of these attacks were mild, five of moderate intensity, five severe, and three were of extreme intensity. Illustrative patients are listed in Table I, in order of increasing severity.

This group includes those patients who had rheumatic attacks in 1938 from whom sera were obtained before the onset of symptoms. They were quiescent rheumatic subjects who had been under our observation for a number of years. They were selected for study at this time because each had contracted a bacteriologically proven streptococcal pharyngitis. All but one showed precipitins during the acute attack to an antigen present in their own serum just before the

from Patients with Acute Rheumatism

Patient	Phase III sera (time after infection)	Potential antigens taken during				Character of attack
		Phase I	Phase II			
			*Early	Middle	Late	
	days					
Bou	30	0	0	0	0	
	32	0	0	0	0	
	36	0	0	0	0	
	39	0	0	0	0	
	40	0	0	+	+	Mild
	43	0	0	+	+	
	47	0	0	0	+	
	51	0	0	0	0	
	65	0	0	0	0	
Ale	47	0	−	0	+	
	49	0	−	0	+	
	51	0	−	0	+	
	54	0	−	0	+	
	56	0	−	0	0	Moderate
	59	0	−	0	0	
	66	0	−	0	0	
	73	0	−	0	0	
	80	0	−	0	0	
	87	0	−	0	0	
Sco	16	0	−	0	0	
	17	0	−	0	0	
	20	0	−	0	0	
	23	0	−	0	+±	
	24	0	−	0	++	
	31	0	−	0	++	Severe
	38	0	−	0	0	
	45	0	−	0	0	
	52	0	−	0	0	
	59	0	−	0	0	
Sut	18	−	−	−	0	Fulminating
	20	−	−	−	+	
	22	−	0	−	++	
	24	−	−	−	++	
	28	−	0	−	+	
	30	−	−	−	0	
	34	−	−	−	0	Recovery
	36	−	−	−	0	
	38	−	−	−	0	
	42	−	−	−	0	
Hah	16	−	−	−	++	Fulminating
	19	−	−	−	+++	
	24	−	−	−	+	
	27	−	−	−	++	
	31	−	−	−	++	
	35	−	−	−	++±	Died (autopsied)

* Early, middle, late = 1st, 2nd, 3rd week after infection.

onset of the attack.   Sera taken at 2 to 3 day intervals showed that the concentration of antigen was maximal just before the symptoms appeared and that it disappeared within 3 days thereafter.   In five cases enough sera were obtained before the onset of rheumatism to establish the fact that the antigens with which the above precipitins reacted were not present during pharyngitis but appeared in phase II.   In one of these cases sera taken during the month preceding pharyngitis were also free of antigen.   The precipitin appeared in each case during the peak of the rheumatic cycle and disappeared with subsidence.   The most intense reactions were observed in sera from the severest attacks.   One individual with rapidly fatal fulminating rheumatism gave the strongest precipitin reaction of all cases studied.

Precipitin Reactions Following Uncomplicated Streptococcus Infections without Rheumatism

All the patients presented in Table I had had hemolytic streptococcus pharyngitis.   The possibility that the precipitin reaction was associated with pharyngitis rather than rheumatism was therefore investigated.   In order to determine the serological behavior of patients who recovered from hemolytic streptococcus without developing rheumatism, two control groups were studied.   These consisted of twenty non-rheumatic subjects and twenty-seven rheumatic subjects with uncomplicated respiratory infections.   Sera obtained during the first 21 days after infection were tested as potential antigens, and sera obtained thereafter as potential antibodies.

The results of these tests were negative except in three patients who developed precipitins 4 to 5 weeks after infection.   In one of these the antibody serum reacted only with serum from the period of acute infection (phase I).   In the other two the antibody sera reacted with serum taken 2 weeks after infection; that is, their serological behavior was similar to that seen in the rheumatic group.   It is of interest that, of these two, one had to be readmitted to the hospital because of abdominal pain accompanied by a high sedimentation rate.   This occurred at the time that the precipitins were present.

The second control group consisted of twenty-seven rheumatic subjects under close observation who contracted hemolytic streptococcus

pharyngitis but escaped all clinical and laboratory signs of rheumatic activity. The sera were assigned to serve as antigens or antibodies

TABLE II

Precipitin Reactions between Potential Antigens and Potential Antibodies in Sera from Rheumatic Subjects with Pharyngitis Who Escaped Recrudescences

Patient	Potential antibodies (time after infection)	Potential antigens taken		
		During acute infection	Following acute infection	
			1st wk.	2nd wk.
	days			
Deu	16	0	0	0
	19	+	+	+
	21	+	+	+
	23	0	+	+
	26	0	+	+
	28	+	+±	+±
	30	0	+±	+±
	36	—	+±	+
	43	+	+±	+
Eas	20	+	+	0
	24	+	+	0
	31	+±	+±	+±
	52	+±	+±	+
Fei	33	0	0	0
	45	+	+	++
	59	0	0	0
Kir	17	0	+	0
	24	+	+	+
	38	0	0	0
	52	0	0	0
Sav	28	+±	+±	+±
	35	+	+	+
	42	0	0	+
	56	+	+	0

just as in the preceding group. The positive results of the precipitin tests are presented in Table II.

The results of these tests were negative in twenty-two cases. These are omitted from the table. The five positive cases developed

precipitins to some substance present during the acute infection and for the next 2 weeks. None of the precipitating sera were positive to phase II (the time corresponding to the symptom-free period) unless they were also positive to phase I (acute infection). These precipitins remained strong in three patients for at least 2 months after infection, in the absence of all symptoms. This type of reaction appeared on further investigation to be distinct from that of acute rheumatism (see page 158).

Precipitin Reactions in Patients with Polycyclic Courses

The data presented so far were confined to the first cycle of acute rheumatism, and in most cases it was clear that the precipitin present at the height of the cycle decreased as disease activity subsided. The next point investigated was whether precipitating sera would recur with repeated cycles of rheumatic activity. It was found that sera taken at the height of cycle II contained good precipitins for phase II antigen. The data are presented in Table III.

It will be noted in Table III that precipitins for phase II antigen recurred during the second cycle of rheumatic activity. It will also be observed that precipitins disappeared from the sera during the periods of remission between cycles of activity. When these remission sera which did not contain antibody were retested as antigens against known positive cycle II antibodies, precipitation was once more observed. The occurrence of antigen during a remission between cycles is illustrated in Table IV.

Antibodies appearing in cycles I and II both precipitated phase II antigen. Furthermore, antibodies appearing in cycle II precipitated antigens developed during the remission between cycles. To demonstrate the identity of the phase II and remission antigens, it was necessary only to show that remission antigens would form a precipitate with cycle I antibody sera, which preceded them in time. The reactions of remission antigens with antibodies which preceded and followed are given in Table IV. The data in Tables III and IV leave no doubt that the antigens of phase II and remission sera are of similar reactivity.

It has often been observed that a rheumatic subject can escape an attack 3 weeks after hemolytic streptococcus infection, only

to develop acute rheumatism some weeks later, at the time that a
second cycle would ordinarily be anticipated. One patient in our
1938 series behaved in this manner. Sera were collected from the
onset of pharyngitis, and the patient was kept under close observa-

TABLE III

*Precipitin Reactions between Sera Taken during the Second Cycle of Acute
Rheumatism and Sera Taken during Phase II*

Patient	Antibodies		Antigens (phase II)		Clinical comment
	Cycle	Date			
San	I	Apr. 19	(Apr. 3) 0	(Apr. 8) +	Acute rheumatic attack
		" 21	+	+	
		" 28	0	0	
					Remission Apr. 30 to May 7
	II	May 10	0	0	
		" 17	0	0	Another cycle
		" 25	+±	+±	
		June 7	0	0	
Low	I	Apr. 23	(Apr. 7) +	(Apr. 14) +	Vague symptoms
		" 28	0	0	High blood sedimentation rate
		May 14	+	+	
		" 19	+	+	
		" 21	0	0	
		" 28	0	0	
		June 4	0	0	
	II	" 18	0	0	Acute rheumatic attack
		" 25	0	+	
		" 27	0	+	
Ter	II	Mar. 7	(Jan. 27) 0	(Feb. 3) 0	Second cycle of acute rheumatism
		" 10	0	+	
		" 12	+	+±	

tion in the hospital. So far as could be determined, she escaped all
evidences of rheumatic activity for 6 weeks after the infection,
and then developed a mild but definite attack of acute rheumatism.
During the first month after infection, it appeared that the patient
had escaped rheumatism and we were unable to detect either antigen

TABLE IV

Precipitin Reactions between Sera Taken during the Remission between Cycles and Sera Taken during the First and Second Cycles

Patient	Antibodies		Antigens (from remission between cycles I and II)		
	Cycle	Date			
Pag	II	May 3	(Apr. 29)		
		" 6	+±		
		" 10	+		
			+		
Lem	I	Mar. 8	(Mar. 28)	(Apr. 5)	(Apr. 7)
		" 12	+±	+±	0
		" 22	+±	+±	+
			+±	+±	+±
	II	Apr. 9	+	+	+
		" 11	+	+	+
		" 13	+	+	+
		" 16	+	+	+
		" 18	+	+±	+±
		" 21	0	+	+
		" 25	+	+	+
		" 27	0	0	0
		" 29	0	0	0

TABLE V

The Late Development of Positive Precipitin Reactions in a Patient with a Delayed Attack of Acute Rheumatism

Antibodies		Antigens					
Stage of disease	Date	Phase I	Phase II		Remission after cycle I of phase III		
		(Jan. 23)	(Jan. 26)	(Feb. 3)	(Feb. 19)	(Feb. 24)	(Feb. 26)
Period usually corresponding to cycle I	Feb. 4	0	0	0			
	" 7	0	0	0			
	" 11	0	0	0			
Period of acute rheumatism	Mar. 2	0	0	0	0	0	0
	" 7	0	0	0	0	0	0
	" 14	0	0	0	+	0	0
	" 21	0	0	0	+	0	0

or antibody. However, she did develop an attack at a time which would ordinarily correspond with a second cycle. Coincident with this, precipitins appeared to serum taken one week before the onset of symptoms. The findings on this patient are given in Table V.

Experiments Dealing with Cross-Reactions between Patients

All the reactions reported so far occurred between sera of different dates from the same patient (hereafter termed homologous sera). The next point investigated was whether these reactions could be duplicated between antigens and antibodies from different patients

TABLE VI *a*

Precipitin Reactions between Homologous and Heterologous Sera

Antigens		Antibodies		Precipitin reaction
Patient	Stage of disease	Type	Stage of disease	
Phi	Phase II	Homologous	Phase III	+±
		Heterologous	Phase III	+±
Sco	Phase II	Homologous	Phase III	++
		Heterologous	Phase III	+
Sci	Phase II	Homologous	Phase III	+
		Heterologous	Phase III	+±
Sut	Phase II	Homologous	Phase III	+±
		Heterologous	Phase III	+±

(heterologous sera). Observations on this point are presented in Table VI *a*.

Table VI *a* shows that heterologous sera gave precipitation reactions of approximately the same intensity as homologous sera when tested against the same known antigens or antibodies. The reactions are therefore not patient-specific. This point was further studied by checking the development of precipitinogen in phase II. The earliest positive antibody serum from each of two patients was tested against potential antigen sera from one of them. In both cases positive reactions occurred with a single antigen serum, taken 14 days after the onset of pharyngitis. The negative antigens were negative

to both homologous and heterologous antibody. The data are summarized in Table VI b.

A similar experiment was run in which the development of precipitin in phase III was determined by tests with three different antigens. As can be seen in Table VIc when a number of antibody sera from one patient were tested against positive homologous and heterologous antigens, differences in the intensity of reaction were apparent. These differences seemed to reflect differences in the strength of the antigens.

TABLE VI b

Development of Precipitinogen in Phase II Sera as Determined by Reactions with Homologous and Heterologous Antibody

Antibodies Phase III	Potential antigens, patient Sci			
	Phase I		Phase II	
Sci (10th day of attack).................	0	0	+	0
Hah (5th day of attack).................	0	0	+±	0

TABLE VI c

The Development of Precipitin in Phase III Sera as Determined by Reactions with Homologous and Heterologous Antigens

Antigens Phase II	Potential antibodies (Sci) Phase III		
	(Mar. 10)	(Mar. 15)	(Mar. 22)
Sci Feb. 26 (weak)........	0	+	±
Sut May 26 (strong)	+±	+±	+

All the antibody sera referred to in Table VI were known to contain precipitin from preliminary positive reactions with their own sera. The next subject of inquiry was whether sera from all patients with acute rheumatism would precipitate known positive antigen sera. To test this point, antigen sera from the two patients in Table VIb were set up against the sera of nine patients with acute rheumatism. Two sera taken some days apart were tested from each patient, to ensure at least one at an appropriate time in the rheumatic cycle. The results are detailed in Table VII.

The series given in Table VII consists of nine unselected cases of

acute rheumatism admitted to the hospital wards some time after the onset of symptoms. No homologous antigens were available. The sera of eight of these patients contained precipitins for heter-

TABLE VII

Precipitin Reactions of Sera from Patients with Acute Rheumatism with Two Heterologous Sera Known to Contain Antigen

Patient (potential antibodies)		Antigens	
		Hah	Sci
Ace	Jan. 8	+	0
	" 24	+±	+
Bar	Mar. 14	0	0
	" 24	+	+±
Bra	Oct. 21	0	0
	" 27	+	+±
DeB	Dec. 27	0	0
	Jan. 3	+±	0
Kau	Dec. 20	+	0
	" 29	+	+
Ros	Mar. 2	+	+±
	" 9	0	+
Smi	Feb. 10	+	0
	" 23	++	+
Ste	Mar. 7	+	+
	" 8	0	+
Wol	Dec. 23	0	0
	" 29	0	0

ologous antigen. The data in Tables V to VII, inclusive, indicate that this precipitation reaction is not patient-specific, but will occur between sera of any rheumatic patients provided that such sera represent appropriate stages of the disease.

Experiments Dealing with the Specificity of the Reactions for Rheumatic Fever

The foregoing data have indicated that during post-streptococcal convalescence, the precipitin reaction occurred only in acute rheumatism with the exceptions discussed. We have just shown that the reaction is not patient-specific. Whether it is disease-specific was next investigated.

First it was ascertained that normal sera were consistently negative to one another and to the sera of acute rheumatism at all stages of disease activity.[2] Then it was found that neither fever nor rapid blood sedimentation paralleled the occurrence of the precipitin reaction. Sera taken during high fevers (associated with tonsillitis, lymphosarcoma, leukemia, tuberculosis, undulant fever) and sera from rapidly sedimenting bloods (associated with post-streptococcal acute nephritis, pneumonia and tuberculosis) were consistently negative to phase II antigen. Reactions did not occur when either antigen or antibody was replaced by serum from other acute febrile diseases.

Another possibility investigated was that this reaction might be related to the non-specific precipitation of pneumococcus C substance reported to occur in acute rheumatism by Tillett and Francis (11). The sera from seven patients with acute rheumatism were found to contain strong precipitins for a 1:50,000 dilution of pneumococcus C. It was also found that, with one exception, the sera of twenty patients who were recovering from streptococcal throat infections but who did not have acute rheumatism, failed to precipitate pneumococcus C substance. These observations supported the possibility that the precipitin reaction might be related to the precipitation of pneumococcus C substance. If such were the case, it should be possible to establish (1) that phase II antigen reacts with anti-pneumococcus sera and (2) that the precipitin of phase III antibody for phase II antigen should be removed by absorption with pneumo-

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[2] The sera of one patient who developed acute rheumatism in the 6th month of pregnancy showed completely divergent behavior. They gave strong positive reactions with all homologous sera, with heterologous rheumatic sera, with normal sera, even with rabbit sera. As the same thing was found to be true for normal pregnancies, this case has been eliminated from the present discussion.

coccus C substance. Experiments on these points were ·therefore set up:—

1. Known positive phase II antigens were tested with sera from four patients with lobar pneumonia and with horse and rabbit anti-pneumococcus sera. These pneumonia sera precipitated C substance but did not precipitate the rheumatic phase II antigens.

2. Positive phase III sera from four cases of acute rheumatism were treated with pneumococcus C. Following absorption, they failed to precipitate C substance but still ·precipitated rheumatic phase II antigens, both homologous and heterologous. An illustrative protocol is seen in Table VIII.

TABLE VIII

The Effect of Absorption with Pneumococcus C on the Precipitation of Phase II Antigens

Patient Cal Antibody Apr. 12	Antigens		
	Pn C	Homologous Cal Mar. 31	Heterologous Low Apr. 14
Unabsorbed...........................	++	+±	+
After absorption with C..................	0	+±	+
After control absorption with saline*........	0	+	+

* Weakening of the pneumococcus C reaction in sera subjected to absorption with saline was observed repeatedly. Simple dilution of serum with saline, how-ever, did not produce this effect.

These two findings indicate that the precipitin reaction of rheumatic fever is not related to the non-specific pneumococcus C reaction common to a number of febrile diseases.

The Relation of the Rheumatic Precipitin Reaction to Streptococcal Derivatives

The next experiment was done to determine whether the precipitin to phase II antigen was an antistreptococcal immune body. This was investigated in two different ways: (A) by testing known positive phase III antibody sera for antibodies to various well known fractions of hemolytic streptococcus; and (B) by testing known positive phase II sera for streptococcus antigens.

Five known positive phase III antibodies were each tested against the following streptococcus fractions: C (group-specific carbohydrate), P (nucleoprotein) and M substance of the same type as the patients'

throat culture taken during phase I.   Three of the sera were negative
to C, and two others to P, indicating that in certain patients anti-C
and anti-P are not involved in the observed precipitin reaction.   The
data are presented in Table IX a.

All of the antibody sera contained anti-M at some time during phase
III; however, the reactions of phase III antibody with M substance

TABLE IX *a*

Precipitin Reactions of Phase III Sera with Streptococcal Fractions and Homologous
Phase II Sera

Antibodies		Antigens			
		Streptococcal			Homologous phase II
		M	C	P	
Ale	May 21	++	0	±	+
Cal	Apr. 12	+±	++	++	+
Kea	" 18	±	0	0	+
Lem	" 9	0	0	0	+
Sut	June 6	++	++	++	++

TABLE IX *b*

The Effect of Absorbing Phase III Antibody with M Substance on the Precipitation of
Phase II Antigen

Patient Sut (type 15 or 17) Antibody	Antigens		
	M-15	M-17	Homologous phase II antigen May 26
Unabsorbed..............................	+	+	+±
Absorbed with M-15.	0	0	+
Absorbed with M-17.....	0	0	+
Control absorption with saline	+	0	+

did not parallel their reactions with phase II antigen (five cases ex-
amined). This suggested but did not prove the independence of
rheumatic precipitin and anti-M. Phase III antibody sera from four
cases were therefore absorbed, each with its own type-specific M
substance (previously determined from throat culture isolated during
phase I) to determine whether the precipitin to phase II antigen
could thereby be removed. Following absorption the phase III

sera no longer precipitated M substance but did still precipitate phase II antigen. A typical experiment is presented in Table IX *b*.

Secondly, known positive phase II antigens were tested with four antistreptococcal therapeutic sera known to contain antitoxin, anti-P and anti-C. These reactions were all negative. Similar tests were performed on antigens from the same patients whose throat infections had been typed, using type-specific anti-M rabbit sera. The results were again negative as seen in Table X.

These findings indicate that the phase II antigen is not one of the four streptococcal products investigated. Phase III sera contain several antibodies to hemolytic streptococcus; but the precipitin to

TABLE X

Precipitation of Rheumatic Antigens by Type-Specific Anti-M Rabbit Serum and Homologous Phase III Serum

Type No.	Antigens		Antibodies	
			Type-specific anti-M rabbit serum	Homologous phase III serum
15	Sut	June 6	0	+
29	Lem	Mar. 3	0	+
30	Cal	" 31	0	+
30	Kea	Apr. 18	0	+
30	Sci	" 19	0	+

phase II antigen seems to be distinct from the bacterial antibodies investigated.

The Nature of Positive Reactions in Rheumatic Subjects Who Escaped Rheumatic Attacks

It was seen in Table II that a few individuals who escaped rheumatic attacks nevertheless developed antibodies in phase III (page 148). These sera precipitated all those which preceded them, not only phase II but also phase I. In other words, the antigen involved was already present during phase I. The question raised by these patients was whether the precipitin of these phase III sera was the same as the precipitin in phase III sera of rheumatic subjects who developed attacks. To throw light on this problem, the sera in

question were tested with known positive antigens of patients who had developed attacks. The results are shown in Table XI.

From Table XI it is seen that the precipitin observed in these two patients was distinct from the precipitin which followed the development of acute rheumatism.

The Nature of Negative Reactions

The next point to be investigated was the reason for the negative reactions observed between pairs of homologous sera taken during recovery from throat infections. In these cases, where both sera were of unknown character, failure to form a precipitate may have been due to the absence of antigen, or antibody, or both; or to

TABLE XI

The Absence of Precipitin Reactions between Known Positive Phase II Antigens and Antibodies from Patients Who Escaped Attacks

Antibodies positive to phase 1		Antigens					
		From phase 1 not followed by attacks		From phase II followed by attacks (heterologous)			
		Deu	Eas	a	b	c	d
Deu	Apr. 11	+±	+±	0	0	0	0
Eas	" 22	+±	+	0	0	0	0

their presence in unsuitable proportions. Accordingly potential antigens and potential antibodies which had been negative to their homologous sera were retested against known positive antibodies and antigens, respectively, to determine which of the essentials of a positive reaction was missing.

A. Negative Reactions in Rheumatic Subjects Who Escaped Attacks.—

*Antibodies.—*The first experiment of this type was set up to retest the precipitating power of two potential antibody sera from four rheumatic subjects who recovered from pharyngitis without developing acute rheumatism. Eight of these sera were tested with four known positive phase II antigen sera. The results shown were negative, indicating that antibodies to phase II antigen were absent from all of eight sera reexamined.

*Antigens.—*Ten potential antigen sera which had been negative to

homologous sera collected about 4 weeks after infection, were retested with known positive rheumatic antibodies. The data are presented in Table XII. Half of these rheumatic subjects, who escaped attacks and who did not produce precipitins, nevertheless developed some antigen which could be precipitated by known positive heterologous antibodies.

TABLE XII

Precipitin Reactions between Apparently Negative Phase II Antigens (from Rheumatic Subjects Who Escaped Attacks) and Known Positive Phase III Antibodies

Potential antigens		Homologous	Antibodies				
			Known positive phase III				
			For	Ale	Kea	Low	Sut
			(Apr. 20)	(May 26)	(Apr. 20)	(June 25)	(June 14)
Ale	Mar. 5	0	0				
All	" 31	0	+				
Car	" 31	0	+				
Pau	Apr. 7	0	0				
Kov	Mar. 25	0		0	0	0	0
Ham	" 10	0		0	0	0	+
Lal	Apr. 18	0		0	+	0	0
Nor	May 14	0		0	0	0	0
Kir	Feb. 26	0		0	0	0	0
McA	Apr. 25	0		+	+	0	+

B. Negative Reactions in Non-Rheumatic Subjects.—

Antigens.—It has been previously shown that some rheumatic subjects who escaped rheumatism following hemolytic streptococcus infections developed antigen without forming antibodies to it. The next experiment was done to determine whether in non-rheumatic subjects who showed no precipitin reaction an antigen was likewise present. Sera from ten patients taken about 2 weeks postinfection were retested with four known positive precipitins from phase III of acute rheumatism.

Of the thirty-two tests, only one gave a positive reaction. The number of cases is too small for final conclusions; but the observations suggest that non-rheumatic subjects do not develop an antigenic substance at a time corresponding to the latter part of phase II.

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DISCUSSION

Although it is not the purpose of the present study to identify the precipitinogen with which the foregoing experiments deal, it is clear that the reaction is distinct from the precipitation of pneumococcus C substance and of certain of the streptococcus antigens. It is, of course, possible that the phenomenon observed does not represent an antigen-antibody reaction.

Whatever its nature may prove to be, as a working hypothesis this precipitinogen may be considered either a primary or secondary antigen. Its absence in sera taken during phase I and its late appearance in phase II would seem to be against its direct bacterial origin, although this cannot be excluded. Another possibility is a secondary antigen such as the precipitinogen described by Hughes in yellow fever (12). It has been shown (13, 3) that in rheumatic subjects who develop acute rheumatism, the appearance of circulating antibodies to hemolytic streptococcus is characteristically late. During this delay streptococcal products are presumably free to react with human tissue constituents. Such an interaction might result in the precipitinogen with which we are dealing.

SUMMARY

1. A precipitin reaction occurs between sera taken just before and shortly after the onset of acute rheumatism.

2. It recurs with repeated rheumatic cycles.

3. Certain properties of the precipitinogen and the precipitin are described.

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OBSERVATIONS ON THE RELATION OF THE EYE TO IMMUNITY IN EXPERIMENTAL SYPHILIS*

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PLATES 9 TO 11

INTRODUCTION

A knowledge of the extent to which the various tissues of the body participate in the acquired resistance which develops during the course of syphilitic infection is essential to our understanding of the mechanism of the syphilis-resistant state. It is well known that the specific resistance which develops in rabbits that have been infected with syphilis is not a property of any one tissue of the host but is shared by other tissues as well, although to a variable extent. Thus the skin certainly acquires a fairly high degree of resistance to syphilitic infection and so also the bones (1). In the case of the eye, however, the situation appears to be different, but the evidence on this point is conflicting and has given rise to some confusion. In this connection three fundamental questions present themselves. First, to what extent does the eye participate in the general resistance which develops during the course of syphilitic infection; second, will a primary focus of syphilitic infection in the eye give rise to a general resistance to the infection such as develops when the primary focus is located in other tissues: and third, does an ocular syphilitic lesion produce a local immunity to syphilis in the affected eye? The experiments reported in this communication were designed to throw light on the first of these questions; we hope to present data on the other two in a subsequent communication.

Several investigators have interested themselves in the general problem of the relation of the eye to immunity in syphilis, and have approached it from the experimental standpoint, but as yet there is no unanimity in the results which have been reported. In any evaluation of experiments dealing with immunity in syphilis it is necessary to take into account certain factors such as the duration of the infection at the time the second inoculation is made, whether or not treatment has been given prior to reinoculation, whether or not the virus used for the second inoculation belongs to the same strain or to a different strain of treponemes, and, finally, the method of reinoculation. Unless due allowance is made for all of these factors erroneous interpretations may easily be made; and it may be profitable, therefore, to consider these factors in some detail before presenting our experimental data.

The duration of the first infection at the time the second inoculation is carried out is important because it has a distinct bearing upon the response of the animal to the second infection. It is now well established that an appreciable amount of time must elapse before the host acquires an effective resistance against syphilitic infection. In the rabbit the time required is somewhere between 45 and 90 days following infection. Second inoculations of syphilitic rabbits with homologous strains of *Treponema pallidum*, carried out prior to the 45th day of the infection, are followed by second infections with lesions, while second inoculations made after the 90th day are not followed by second infections with lesions. It is evident, therefore, that at some time between the 45th and the 90th day of the infection the acquired resistance of the syphilitic rabbit reaches the level where it protects the animal against a second attack of the disease; it follows from this fact that reinoculation experiments designed to test the acquired immunity of any tissue of the rabbit should not be carried out until after the 90th day of the infection. If they are performed before that time they are of no value for that particular purpose.

The question whether or not the animal has been treated prior to the second inoculation, and the time at which such treatment was instituted, has also to be taken into consideration because it has been well established that treatment of the rabbit with arsphenamine prior

to the 45th day of the infection leaves the animal completely susceptible to a second infection, whereas if treatment is postponed until after the 90th day the resistance which has been built up during the preceding period is not abolished by treatment but persists for some time thereafter and is sufficient to protect the animal against a second attack of the disease. It is clear from this fact that reinoculation experiments carried out on rabbits treated before the 90th day of the disease cannot be taken into consideration in determining whether or not particular tissues of those animals, such as the cornea, are refractory to a second inoculation of syphilitic virus. Attention can only be paid to those reinoculation experiments in which treatment is begun after the 90th day of the infection.

The identity of the strain used for the second inoculation must also be taken into consideration in evaluating all experiments dealing with immunity in syphilis because it has been demonstrated that the resistance which is acquired by rabbits during the course of a syphilitic infection is largely effective against the homologous strain of *T. pallidum* and not against heterologous strains of that organism. Accordingly, all experiments in which heterologous strains are used for reinoculation must be excluded if one is seeking to determine the participation of this or that particular tissue in the immune reaction of the rabbit.

Since in this instance we are seeking to ascertain the results obtained with a particular method of reinoculation (intraocular), obviously, for the sake of comparison, we need consider only those reinoculation experiments in which that particular method has been employed. The experiments of that sort which have been reported in the literature have been analyzed by us, keeping in mind the points mentioned previously, namely, time of reinoculation, time of treatment, and identity of the strain used for reinoculation. On the basis of what is known about immunity in syphilis, as set forth above, we felt impelled to exclude from consideration all those experiments in which reinoculation was performed before the 90th day of the infection, as well as those in which the strain of syphilitic virus used for reinoculation was different from the one used for the first inoculation. When the experiments dealing with the intraocular reinocula-

tion of syphilitic rabbits that have been reported in the literature were subjected to such criteria, some of them had to be rejected. Those that met the criteria satisfactorily are summarized in Tables I and II.

TABLE I

Summary of Results of Ocular Reinoculation of Untreated Syphilitic Rabbits Carried Out 90 Days or More after the First Inoculation

First inoculation scrotal or testicular, second inoculation ocular.

Author	Interval between 1st and 2nd inoculation	Results of 2nd inoculation			Positive
		Positive	Doubtful	Negative	
					per cent
Frei (2)............	½–1½ yrs.	4	—	22	15.4
Adachi (3)...........	95–361 days	9	2	15	34.6
Frei and Hahn (4).....	3½ mos.–2 yrs.	5	2	7	35.7
Breinl (5)...........	5 mos.	—	—	5	0
Total............		18	4	49	36

TABLE II

Summary of Results of Ocular Reinoculation of Syphilitic Rabbits Given Antisyphilitic Treatment 90 Days or More after the First Inoculation

First inoculation scrotal or testicular, second inoculation ocular.

Author	Interval between 1st inoculation and treatment	Results of 2nd inoculation			Positive
		Positive	Doubtful	Negative	
					per cent
Tomasczewski (6).............	90–105 days	5	—	2	71
Uhlenhuth and Mulzer (7)......	119 days	—	—	2	0
Frei (2).................	7 mos.–2¼ yrs.	3	—	20	13
Adachi (3)................	120–363 days	0	2	7	0
Mulzer and Nothhaas (8).......	105–271 days	6	—	5	54
Gaibissi (9)...............	5–13 mos.	8	1	4	61
Total...............		22	3	40	55

It is evident from the data presented in these tables that there is no unanimity in the results obtained by the different investigators. Nor is it clear from the protocols of their experiments why there should be such differences as appear in the tables. Because of the variations

in the results obtained by other workers we have thought it advisable to carry out some experiments along the same lines under standard conditions and with a single strain of *T. pallidum* that has become well adapted to the rabbit through propagation in that species over a period of years. The purpose of this communication is to present the results of those experiments.[1]

Technique

Inoculum.—The Nichols strain of *T. pallidum* was used in all experiments, both for primary inoculations and for reinoculations. Virus emulsions in physiological salt solution, prepared by grinding syphilitic testicular lesions in a mortar and centrifuging the emulsion to throw down the larger tissue particles constituted the inoculum in every instance.

Treatment.—All test animals were given 5 intravenous injections of arsphenamine. Each dose was 20 mg. per kg., and the drug was given at intervals of one week. Treatment was begun from 163 to 217 days after the first infection.

Intracorneal Inoculation.—Under ether anesthesia, an eye speculum was placed beneath the lids and nictitating membrane, and the episclera was grasped above with fixation forceps. Using a No. 26 gauge needle and a tuberculin syringe, the needle was thrust between the lamellae of the cornea until the bevel was fully in the corneal parenchyma, and an intracorneal injection of 0.1 cc. of the virus was made, care being taken not to enter the anterior chamber. The injected material spread out as an opaque film between the layers of the cornea and covered as a rule about two-thirds of that structure. Occasionally, there was either some tearing of the conjunctiva with deposition of the virus on the torn surface, or accidental puncture of the anterior chamber, with deposition of the virus in the anterior chamber.

The eyes of the animals were examined twice weekly for the first 6 months, and once weekly thereafter, and the reactions occurring were carefully noted. In recording the severity of the reactions a numerical system was used. The degree of ciliary inflammation, of corneal reaction, and of the inflammatory reaction in the iris were each given a numerical rating. The extreme reactions were noted as 4, and the less extensive reactions given a correspondingly smaller number. The total reaction in the eye was recorded as the sum of the individual reactions. In none of the animals were reactions in the vitreous, orbit or fundus noted.

Cutaneous Inoculation.—All injections to test the susceptibility of the skin were made by intracutaneous inoculations, in shaved areas on the back, of 0.1 cc. of the virus emulsion.

Animals.—The rabbits used belonged to all the commoner breeds, and were not limited to any particular variety.

EXPERIMENTS

Experiment 1.—12 rabbits were inoculated intracutaneously on the back with active syphilitic virus and in all of them characteristic lesions developed at the site of inoculation. Treatment was begun 163 days after the inoculation. Second inoculations were carried out on the surviving 11 animals 337 days after the first inoculation, or 143 days after the last dose of arsphenamine. Into the left cornea of each of these animals was injected 0.1 cc. of active syphilitic virus emulsion, and into the right cornea an equal amount of an emulsion prepared from a normal rabbit testis. 12 normal rabbits were inoculated intracorneally with the same syphilitic virus in the left eye and with the same normal rabbit testis emulsion in the right eye. Of these, two died prematurely so that only 10 remained for comparison with the test animals. The results of the experiment are shown in Table III.

It will be seen from Table III that in the corneas of 9 of the 10 normal animals inoculated with syphilitic virus characteristic syphilitic lesions developed within the customary incubation period. In 6 of the 11 "immune" animals characteristic syphilitic lesions made their appearance in the eye inoculated with living virus. In none of the eyes inoculated with the normal testicular emulsion did any lesions develop other than an immediate reaction which promptly subsided.

The average incubation period of the lesions developing in the "immune" group was slightly greater than that of the control group, but once they had made their appearance the lesions in the eyes of the supposedly immune animals persisted for a longer time, and showed a marked tendency toward recurrence.

The character of the corneal lesions which developed after the intracorneal injection of syphilitic virus was essentially the same in both the normal and in the "immune" rabbits. There was usually first a faint superficial vascular invasion coming in from the limbus (Fig. 1). In some rabbits this quickly disappeared, while in others it persisted and became part of the general ocular picture. After an incubation period of varying length, the keratitis as a rule took one of four courses. The first type observed consisted of an increase in the superficial vascular invasion (Fig. 2), quickly followed by definite

infiltration of the cornea (Figs. 3 and 4).   The early infiltrates in the cornea frequently had no relation to the actual site of injection.   At times they appeared 3 to 4 mm. away from the site of injection, while

TABLE III

Reinoculation of Eyes of Treated Syphilitic Rabbits

First inoculation intracutaneous, second inoculation intracorneal.   Treatment begun 163 days after first inoculation.   Second inoculation 143 days after completion of treatment.

	Rabbit No.	Occurrence of lesion	Incubation period	Maximum intensity of eye lesion	Duration of eye lesion	Recurrence of eye lesion	Occurrence of metastatic eye lesions	Total period of observations
			days		mos.			mos.
Immune group	35-28	Pos.	103	7	26	0	0	26
	35-22	"	89	7	2	4	0	15
	35-20	"	30	8	Eye enucleated	—	—	11
	37-96	"	36	8	6	0	0	15
	37-46	"	105	5½	1½	1	0	24
	37-42	"	30	8	20	1	0	26
	37-43	Neg.	—	—	—	0	0	5
	37-47	"	—	—	—	0	0	6
	38-27	"	—	—	—	0	0	4
	37-91	"	—	—	—	0	0	24
	35-03	"	—	—	—	0	0	26
Control group	40-34	Pos.	130	5	2½	0	0	26
	40-36	"	89	7	4½	0	0	37
	40-37	"	89	9	1½	2	0	15
	60-41	"	67	7	5	0	0	49
	40-42	"	53	5	1½	1	0	49
	40-44	"	22	6	4	0	0	15
	40-45	Neg.	—	—	—	0	0	44
	40-39	Pos.	15	7	4½	2	1	49
	40-39	"	?	7	6	0	1	24
	40-47	"	22	8	19	0	1	32
					Summary			
Immune group	11 animals, 7 pos 4 neg		7.2 ave.		11.1	In 3 animals	None	16.5 ave.
Control group	10 animals, 9 pos 1 neg		6.8 ave.		5.1	In 3 animals	In 3 animals	34.3 ave.

in other animals the first infiltrates appeared exactly at the site of the corneal puncture. The vascular invasion at first was entirely from the superficial conjunctival vessels, but about 3 months after inoculation deep vascular invasion occurred from the perforating vessels.

The picture gradually progressed to a typical generalized interstitial keratitis—ciliary congestion and hazy infiltrated cornea, becoming almost opaque, associated with vascular invasion of the cornea, both deep and superficial (Fig. 5). There was frequently an associated iritis, with contraction of the pupil, marked dilatation of the iridic vessels, and occasionally a transitory hypopyon (Fig. 6). The iritis was usually evanescent and recurring in character, the attack rarely persisting over one week. This type of reaction represented approximately 40 per cent of the ocular lesions of both normal and immune animals.

The second type of lesion consisted of a yellowish, elevated nodule appearing usually at the limbus (Fig. 7). Following the appearance of this nodule, diffuse infiltration developed in the adjacent cornea and within 2 weeks the picture was one of generalized interstitial keratitis, the lesion apparently spreading out from the primary corneal nodule (Fig. 8). With the exception of the nodule, the final picture was similar to the keratitis in the first type of reaction, the general picture consisting of the yellowish, elevated nodule at the limbus together with ciliary congestion, hazy, infiltrated, steamy cornea, and frequent short attacks of iritis—similar to those already noted. Approximately 50 per cent of the rabbits exhibited this type of ocular reaction.

The third type of lesion was similar to the second, with the exception that the yellowish, elevated papule was situated in the center of the cornea. There was associated ciliary congestion and a rapid spread of the keratitis until over two-thirds of the cornea was involved (Fig. 9). As this central type of lesion progressed, there was some weakening of the central cornea with the formation of a low grade corneal staphyloma, which flattened out as the lesion subsided. This type of reaction was observed in 4, or approximately 7 per cent, of the rabbits injected.

The last type of reaction likewise occurred in the center of the cornea and consisted of a gradually developing deep central haze. In one rabbit this had the appearance of a deep punctate keratitis, while in 2 others the central infiltration was uniform. There was a remarkable absence of both ciliary congestion and corneal vascularization. This peculiar avascular reaction was observed in 2 normal animals and one immune animal.

In two rabbits, in which the anterior chamber was accidentally punctured, the low grade iritis was complicated by the appearance of small whitish nodules at the pupillary border, similar in appearance to the syphilitic papules occasionally observed clinically in syphilitic iritis.

The only ocular complication of any note observed was the development of glaucoma with buphthalmos in 4 rabbits. This was in no way different from the glaucoma and buphthalmos occasionally observed in non-syphilitic experimental ocular lesions in the rabbit, and its possible relation to syphilis has already been discussed in a report by Beckh (10).

The course of the lesions in the two groups was essentially the same, except that in the immune group the lesions tended to recur more often than in the normal group. The high percentage of successful second inoculations encountered in the corneas of supposedly immune animals led us to carry out another experiment along similar lines except that in this experiment reinoculations were made simultaneously into one cornea and the skin of the back of each of the test animals. These intracutaneous inoculations were added in order to determine whether or not the test animals were "immune" to syphilis as judged by direct inoculation into the skin. The details of the experiment follow.

Experiment 2.—A series of 25 rabbits was inoculated intratesticularly with active syphilitic virus. In all of them characteristic orchitis developed. All were treated with arsphenamine, treatment being commenced 170 days after the first inoculation. Of this series 14 animals survived and were available for reinoculation which was carried out 344 days after the first inoculation and 140 days after treatment was completed. Reinoculations were made by inoculating 0.1 cc. of virus emulsion into one cornea and a like amount intracutaneously in a shaved area in the lumbar region. For controls 16 normal animals were inoculated in the same way with the same virus. Of those, 15 survived. The results of this experiment are shown in Table IV.

As will be seen from the table, all of the test animals were refractory to a second inoculation of homologous virus inoculated into the skin but in 9 of them syphilitic lesions developed in the cornea after an incubation period which was twice as long as that of the corneal lesions in the normal animals. The corneal lesions developing in the test animals in this experiment were of shorter duration, on an average,

TABLE IV

Simultaneous Reinoculation of Eyes and Skin of Treated Syphilitic Rabbits

First inoculation intratesticular, second inoculation intracorneal and intra-cutaneous. Treatment 170 days after first inoculation. Reinoculation 140 days after completion of treatment.

	Rabbit No.	Result of reinoculation		Incubation period		Maximum intensity of eye lesion	Duration of eye lesion	Recurrence of eye lesion	Occurrence of metastatic eye lesions	Total period of observation
		Eye	Skin	Eye	Skin					
				days	days	mos.				mos.
Immune group	43-09	Pos.	Neg.	28	0	7	4½	1	0	13½
	43-28	"	"	33	0	4	12	0	0	13½
	43-13	"	"	156	0	2½	1⅔	0	0	13½
	43-17	"	"	124	0	1½	¼	2	0	13½
	43-24	"	"	180	0	3	¾	1	0	13½
	43-34	"	"	33	0	7	11	0	0	13½
	43-35	"	"	144	0	2½	1	0	0	13½
	43-27	"	"	151	0	1½	?	0	0	5
	43-45	"	"	180	0	4½	¾	1	0	13½
	43-16	Neg.	"	0	0	0	0	0	1	10
	43-18	"	"	0	0	0	0	0	0	13½
	43-20	"	"	0	0	0	0	0	0	3½
	43-25	"	"	0	0	0	0	0	0	11
	43-21	"	"	0	0	0	0	0	0	13½
Control group	45-64	Pos.	Pos.	45	21	3	1⅗	1	0	10
	45-65	"	"	28	21	7	7½	0	0	9¼
	45-67	"	"	187	13	1	½	0	1	12¼
	45-68	"	"	28	21	7	7½	0	0	12¼
	45-70	"	"	36	13	4	11	1	0	12¾
	45-71	"	"	41	13	6	9	0	0	12¾
	45-72	"	"	73	13	3	9	0	0	12¾
	45-73	"	"	14	28	6	9	0	0	12¾
	45-74	"	"	34	28	2	8	0	0	8
	45-76	"	"	48	13	5	¾	2	0	12¾
	45-77	"	"	34	21	7	7½	0	0	12¾
	45-78	"	"	28	21	5	7½	1	0	12¾
	45-80	"	"	152	13	2	4	0	0	12¾
	45-36	"	"	152	21	2½	6	0	0	12¼
	45-66	"	"	34	21	5	8	0	0	12¾
Summary										
Immune group	14 animals	9 pos. 5 neg.	0 pos. 14 neg.	114 ave.	0	3.7	4	5	1	12 ave.
Control group	15 animals	15 pos. 0 neg.	15 pos. 0 neg.	62 ave.	17.6 ave.		6½	5	1	12 ave.

than those which developed in the control animals, although a few were of longer duration.

This experiment reveals clearly the difference in the extent to which the two tissues, cornea and skin, share in the refractory state which develops in rabbits during the course of syphilitic infection, when the test is made by reinoculating treated animals simultaneously in both the cornea and the skin. Under the circumstances the cornea is clearly less refractory than the skin and must be assumed, therefore, to share to a lesser extent in the immune process.

In some of the experiments recorded in the literature the reinoculations have been made into the anterior chamber instead of the cornea. In an experiment carried out by one of us in association with Turner, which has never been reported in full but has been referred to elsewhere (11), 4 treated "immune" rabbits reinoculated with homologous virus by injection into the anterior chamber failed to show any lesions in the eyes. This result made it seem advisable to carry out a similar experiment on a larger scale in order to determine if the method of ocular inoculation might play a rôle in the occurrence or non-occurrence of lesions in the "immune" animals. Accordingly in a third experiment this question was put to the test.

Experiment 3.—A series of 20 rabbits was inoculated intratesticularly with active syphilitic virus. In all of them characteristic orchitis developed. All were treated with arsphenamine, treatment being commenced 217 days after the first inoculation. Of this series 18 animals survived. These were reinoculated into the anterior chamber 363 days after the first infection and 146 days after the last dose of arsphenamine was administered. The aqueous was removed with a hypodermic syringe under ether anesthesia and then replaced by 0.1 cc. of the virus emulsion, the needle being left in place and the syringes shifted. The results of this experiment are shown in Table V.

As will be seen from Table V, lesions developed in the eye in 15 of the 18 normal animals and in 12 of the 18 test animals. The average incubation period of the lesions in the supposedly immune animals was about three times as great as that of the lesions in the control animals, while the duration of the lesions in the test animals was distinctly less than that in the controls. It is evident from this experiment that the resistance of the supposedly immune animals to a second inoculation of homologous virus introduced into the anterior chamber was lacking in a very high proportion of the animals.

TABLE V

Reinoculation of Anterior Chamber of Treated Syphilitic Rabbits

First inoculation intratesticular, second inoculation into anterior chamber. Treatment begun 217 days after inoculation. Second inoculation 112 days after completion of treatment.

	Rabbit No.	Occurrence of eye lesion	Incubation period (days)	Maximum intensity of eye lesion	Duration of eye lesion (mos.)	Recurrence of eye lesion	Occurrence of metastatic eye lesions	Total period of observation (mos.)
Immune group	45-53	Neg.	—	—	—	—	—	12
	45-88	Pos.	76	5	3½	0	0	12
	45-89	"	97	4	1½	0	0	12
	45-92	"	90	5	2¾	0	0	12
	45-96	"	53	8	2	1	0	12
	46-10	Neg.	—	—	—	0	0	12
	46-11	"	—	—	—	0	0	12
	46-13	"	—	—	—	0	0	12
	46-14	Pos.	76	3	1½	0	0	12
	46-16	"	61	5	¾	1	0	12
	46-17	Neg.	—	—	—	0	0	12
	46-18	"	—	—	—	0	0	12
	46-19	Pos.	39	5	2	1	0	12
	46-21	"	97	6	1¼	0	0	12
	46-22	"	132	3	¾	0	0	12
	46-23	"	41	7	3	0	0	12
	46-24	"	53	5	2½	0	0	12
	46-25	"	53	4	2½	1	0	12
Control group	48-70		26	6	3½	0	0	12
	48-69		27		3½	0	0	12
	48-23	Neg.	—		—	—	—	7
	48-71		21	6	4¼	0	0	12
	48-72		19	7	4½	0	0	12
	48-73		19	6	3¼	0	0	12
	48-74		19	5	3¼	1	0	12
	48-32		19	3	2½	0	0	12
	48-33	Neg.	—	—	—	—	—	12
	48-11		19	9	¾	0	0	12
	48-35		27	4	3	0	0	12
	48-36		41	3	2½	1	0	12
	48-37		32	5	4	0	0	12
	48-38		19	5	4¼	0	0	12
	48-39		27	8	4	0	0	10
	48-42	Neg.	—		—	—	—	12
	48-45		34	4	4	0	0	12
	48-40		27	2	3½	1	0	12
Summary								
Immune group	18 animals	12 pos. 6 neg.	72 ave.	5	2 ave.	4	0	
Control group	18 animals	15 pos. 3 neg.	25 ave.	5.2	3⅗ ave.	3	0	

DISCUSSION

These experiments demonstrate that in the rabbit the cornea does not share to the same extent that other tissues, notably the skin, share in the acquired resistance which develops during the course of syphilitic infection. In some of the animals the cornea seems to achieve complete protection against homologous strains of *T. pallidum* but in the majority of cases it does not do so, although a considerable lengthening of the incubation period was observed in most of the "immune" animals in which a lesion developed in the cornea following reinoculation. This prolongation of the incubation period is doubtless the expression of a slight amount of resistance which the cornea acquired, but it must represent a small amount indeed.

In both the normal and the "immune" groups immediate reactions were observed at the site of inoculation, due to the injection of foreign material, but these immediate reactions in the test animals were not more violent than those occurring in the control animals, nor was any tendency to accelerated reaction observed in the test group. There is no evidence in these experiments, therefore, to indicate that the corneas of the "immune" rabbits were allergic to syphilitic virus.

The results of these experiments offer a possible explanation for the well established clinical observation that the interstitial keratitis of congenital syphilis is prone to recur in patients with that condition. On the basis of the behavior of rabbits one might infer that this tendency to recur is due to failure of the cornea of the patient with congenital syphilis to participate in the general defense reaction against the infection. Such an inference would suffice to explain the tendency of such patients to show repeated relapses of interstitial keratitis, but would not explain the fact that this condition is singularly resistant to antisyphilitic treatment. Some ophthalmologists, notably Igersheimer and Derby, have sought to explain the proneness of interstitial keratitis to relapse on the basis of allergy, and have supposed that the corneas of such patients become allergic to the treponemes or their products. The experiments reported in this communication lead no support to this view. Furthermore attempts made by us to sensitize the corneas of normal rabbits to *T. pallidum* by the preliminary injection of killed suspensions of that organism were entirely fruitless, and actively motile specimens of *T. pallidum* could be

demonstrated in the macerated corneas of the test animals in which lesions developed. There is therefore no need and indeed no ground for invoking a state of allergy to explain the findings in rabbits. It is possible, of course, that the situation may be quite different in man, and that the human cornea under certain conditions may become allergic to the treponemes of syphilis or their products, but there is no direct evidence that such is the case, and it is certainly not necessary to assume that interstitial keratitis is due to anything other than the invasion of a susceptible tissue by the organisms themselves. That the treponemes may invade the cornea in cases of congenital syphilis has been proved in one case by Igersheimer who succeeded in demonstrating them in the cornea of a 14 year old girl with congenital syphilis. It is obvious that there is not likely to be a large enough series of such observations to give us an inkling as to whether such a result is the exception or the rule.

We are not in a position to offer a satisfactory explanation as to why the cornea of the syphilitic rabbit should be so much less refractory to a second inoculation of syphilitic virus than other tissues such as the skin, for example. One instantly thinks of the absence of a blood supply as a possible explanation for the failure to share in the general resistant state. It may well be that owing to this absence of a blood supply the corneal cells either do not receive an antigenic stimulus which is sufficient to cause them to become immune themselves, or that they do not receive from the circulation enough of the hypothetical syphilis antibody to endow them with resistance to syphilitic virus, but it is not possible at the present time to say which of these two explanations is the correct one, and it will be necessary to accumulate more data before we shall be in a position to judge between them.

SUMMARY AND CONCLUSIONS

Three experiments are reported in which an attempt has been made to determine the extent to which the eye participates in the general resistance which develops in rabbits during the course of syphilitic infection. Rabbits treated with arsphenamine well after the period when they would be expected to be immune to intratesticular or intracutaneous inoculations were reinoculated with the homologous

rain of *T. pallidum*, the organisms being injected into either the
rnea itself or the anterior chamber. Altogether in the three ex-
riments 43 presumably immune animals were injected, 25 into the
rnea itself and 18 into the anterior chamber. 41 normal animals
ere used as controls. In the immune animals 27 or 62 per cent
iowed lesions in the cornea. 14 of the 43 test animals were inocu-
ted simultaneously in the cornea and in the skin of the back. In
of these 14, lesions developed in the cornea although no lesions de-
eloped in the skin.

The lesions developing in the corneas of the "immune" animals had
longer incubation period on the average, were often of longer dura-
on, and in some instances were more severe than the lesions develop-
ig in the control animals. In the case of some animals, also, they
howed a greater tendency to recur. The immediate reactions in
ioth the normal and the "immune" animals were entirely comparable
.nd there was no evidence of an accelerated reaction in the test
.nimals.

It is concluded that the eye of the syphilitic rabbit does not share
.o the same extent as other tissues in the general resistant state which
levelops in that animal during the course of syphilitic infection.
Possible explanations for this finding are discussed.

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EXPLANATION OF PLATES

These figures are designed to be viewed with the standard Bausch and Lomb hand stereoscope.

PLATE 9

FIG. 1. Initial vascular invasion of the cornea from superficial conjunctival vessels.

FIG. 2. More marked vascular invasion of the cornea.

FIG. 3. Early infiltration of the cornea.

FIG. 4. More advanced infiltration of the cornea.
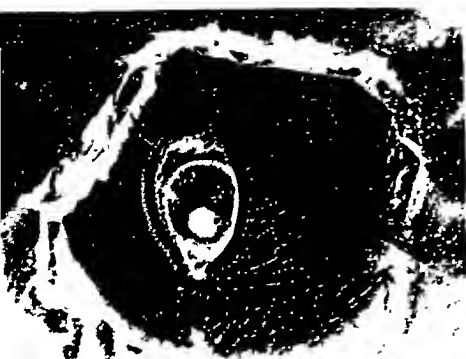
FIG. 9. Advanced interstitial keratitis with iritis.
FIG. 10. Central corneal infiltration without vascularization.

PLATE 11
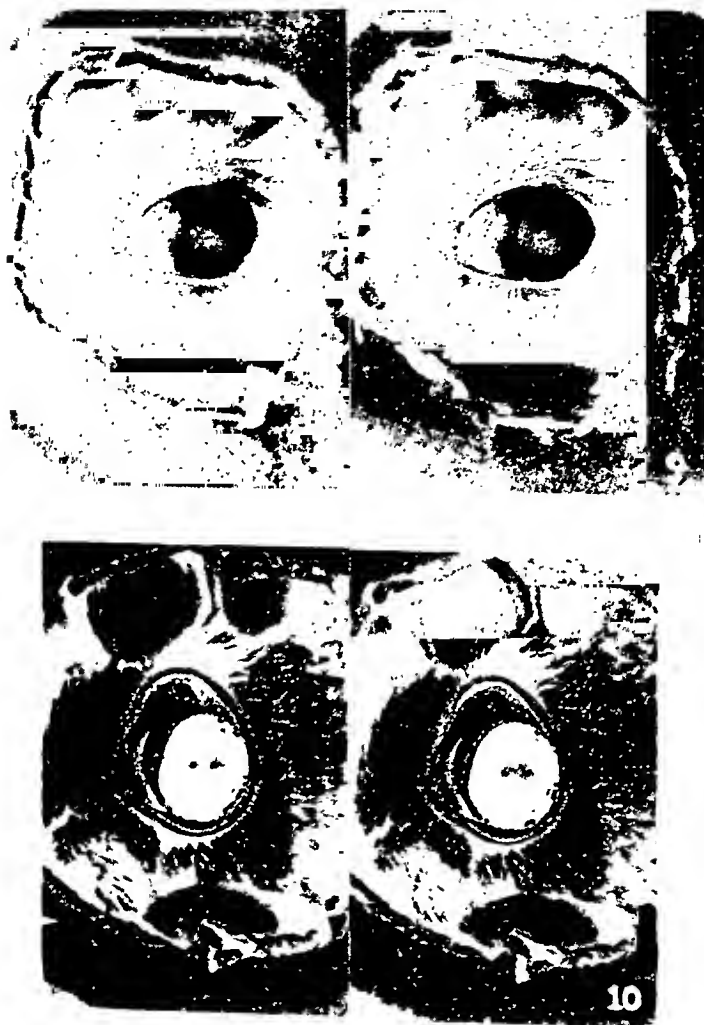
(Chesney *et al* : Relation of eye to immunity in syphilis)

CORRECTION

Page 435, Table X, third entry under s_{xy}, for $\underline{1.2}$ read $\underline{3.3}$

A VIRUS DISEASE OF CATS, PRINCIPALLY CHARACTERIZED BY ALEUCOCYTOSIS, ENTERIC LESIONS AND THE PRESENCE OF INTRANUCLEAR INCLUSION BODIES

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PLATE 18

During the fall of 1937 an epizootic disease broke out among our laboratory stock of kittens. The signs presented were practically limited to anorexia, weakness and occasional vomiting. The outstanding clinical findings were an elevation of temperature and a fulminating, early leucopenia which not infrequently progressed to the point where no leucocytes remained in the circulating blood. In the majority of animals death usually occurred from the 2nd to the 4th day after the appearance of outward signs of illness. In most cases at autopsy there was little of note except a slight or moderate congestion of portions of the small intestine and a very soft, almost fluid marrow in the femur. The salient features upon microscopic examination of sections were a non-inflammatory lesion of the intestine with conspicuous intranuclear inclusion bodies in the cells of the mucosa, a marked aplasia of all or most of the cellular elements of the bone marrow, and lesions in the spleen and lymph nodes in which certain mononuclear cells containing intranuclear inclusion bodies were observed. No single bacterial species could be cultivated with regularity from these kittens. Our experience with older animals was limited, but when the infection did occur among them it appeared to present the same general aspects with occasionally certain modifications which will be subsequently described.

The clinical and pathological findings were so constant and so striking that a systematic attempt was undertaken to study the disease.

The literature dealing with epizootic diseases of cats contains frequent reference to "distemper," a term which has been applied to nearly all feline infections of a highly contagious nature. This so called distemper has been often described as protean in its manifestations. In recent years there has been a tendency on the part of a few observers to apply the term distemper of cats to an infection which is predominantly respiratory in nature and to designate as "infectious enteritis" a disease with marked gastro-intestinal symptoms and lesions.

Verge and Cristoforoni (1) described a gastro-enteritis of cats which they believe they were able to transmit to other cats by a filtrate of organs from the infected animal. Hindle and Findlay (2, 3) isolated a variety of bacteria from a disease of cats with gastro-intestinal symptoms which they called distemper. These organisms failed to reproduce the disease upon injection, but the typical infection followed injection with filtrates of nasal washings or suspensions of various organs. Urbain (4) stated that he was able to transmit a gastro-intestinal infection of cats by the inoculation of filtrates. Leasure, Lienhardt and Taberner (5) also describe a similar disease which they believe they passed by means of filtrates. Dalling (6) in a paper entitled "Distemper of the cat" described an enteric infection which was transmissible by filtrates. He concluded despite the title of his paper that the disease with which he worked is an entity distinct from the disease or group of diseases in which respiratory symptoms predominate. In discussing Dalling's paper, Findlay (6) asserted that all these fatal epizootics of cats commonly called distemper are due to a single virus and that the variations encountered in the symptomatology and pathology are to be attributed to differences in the type of secondary invaders. Gray (6) in his discussion of the same paper stated that "infectious enteritis is a disease clinically diametrically opposite to distemper."

While the work to be reported was being terminated a note by Lawrence and Syverton (7) appeared wherein they described a spontaneous, infectious "agranulocytosis" in the cat, which as nearly as we can judge from their published data is probably identical with the disease which we have investigated.

The obvious · confusion in respect to etiology and classification appears to depend in our opinion upon the fact that no pathological characteristics have been described by which the condition or conditions may be securely recognized or differentiated. In the disease which we have encountered the changes briefly noted above, and which will be subsequently described in detail, furnish we believe, adequate criteria by which this particular infection may be identified.

Clinical Course

The entire clinical course of the disease has been studied systematically in the case of 10 cats, whereas in 21 others the disease was identified by at least one leucocyte count in addition to post mortem findings. In 19 other animals the diagnosis was made solely on gross and histological post mortem evidence.

A high mortality has been observed among those animals in which definite signs of illness become apparent. Thus among a series of 12 cats exhibiting all the criteria by which the disease may be recognized during life 2 only recovered. Of the 10 which died,[1] the autopsy findings both in the gross and microscopically revealed all the pathological changes which we have found to be associated with the disease from a study of the 50 cases mentioned above and which include these 12 animals. This series illustrative of the mortality rate comprises relatively few animals, since those which were killed during the acute stage and those in which observations on the course of the infection made during life were incomplete or lacking, have been excluded.

Although it is clear that the disease terminates fatally in a large percentage of cases when it has been sufficiently severe to produce easily recognized sickness in the cat, a few animals which have been studied have presented no other indication of illness than a moderate leucopenia. We believe these represented true infections which would not have been recognized unless routine white blood cell counts had been carried out. It is therefore entirely possible that under natural conditions many cats may undergo a mild, inapparent infection.

Following either spontaneous infection or the inoculation of infectious material,[2] after a variable incubation period, the animals refuse food and drink, become depressed and weak and tend to lie on the bottom of the cage, but they are usually responsive, stand, purr and rub up against the side of the cage when approached and called or mildly stimulated until 2 or 3 hours before death. Never has any excitement been noted. Death is usually rather sudden and unexpected, preceded by a few minutes of fibrillary twitching, terminating in clonic convulsions in those which were observed closely. Manifest signs of disease usually persist only

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[1] One of these was killed *in extremis.*

[2] A section on the experimental transmission of the disease will be found on page 339.

2 days before death, whereas moderate leucopenia and sometimes slight elevation of temperature, which we will describe in detail, are noted 3 or 4 days previous to death.

Vomiting.—A scant quantity of a bile-tinged, glary, mucoid vomitus was usually found in the cage from time to time during the period of acute illness.

Diarrhea.—Diarrhea was the exception rather than the rule, bloody stools were never observed, and solid fecal material was regularly found in the colon at autopsy with one exception. No tests have been made for occult blood.

Coryza and Conjunctivitis.—In a few instances purulent conjunctivitis occurred either early or late in the course of the disease and in a slightly larger percentage signs of coryza were noted, usually preceding the more acute symptoms. These were interpreted as accidental or secondary to the principal disease.

Temperature.—In our experience the temperature of the normal cat has been found to range from 101.0° to 102.5°F. Although this disease is accompanied by an elevation of temperature, the fever is seldom of an extreme degree. Thus in every instance but one, in those animals followed clinically, the temperature rose above 103°F. sometime during the course of the infection, but only twice was a temperature above 105°F. noted and that in each case was below 106°. The maximum in the majority of instances was between 103.6° and 104.6°F. and occurred 1 or 2 days before death, most frequently on the day before death. In many instances the temperature rose to 103°F. or above on the day leucopenia was first observed, but not infrequently leucopenia was noted 1, and exceptionally 2, days prior to the elevation of temperature. In the few animals where the temperature was taken just prior to death it was found to be subnormal.

Table I gives a summary of the temperature changes in a group of fatal cases, and Tables II, IV and V illustrate the course of the fever in 2 fatal cases and in one which recovered.

White Blood Cells.—Among 80 counts of the leucocytes in the blood of 40 cats in good health we have encountered only three below 10,000 per c.mm. of blood. Of these two were 9,000 and 8,900 and the third 5,000. The last in all probability represented an error in technique, since counts on each of the following 3 days on the same cat were all found to be above 10,000. The average of these 80 counts was 17,800; the highest being 46,400. These data agree in general with those of other investigators (8), one of whom however has recorded one count as low as 6,000.

Leucopenia, as we have stated, is a constant characteristic of the disease as we have seen it and affords an important diagnostic criterion. Tables I, II, III, IV and V demonstrate this striking diminution of the leucocytes in the circulating blood.

In the great majority of infected cats in which a white blood cell count was made on the day of spontaneous death it was below 1,000 per c.mm. of blood and

in no case was it above 3,000. In 2 animals no leucocytes of any kind could be found on the day prior to death and this undoubtedly would have been more frequently observed had not the animal been sacrificed when counts in the neighborhood of 100 to 300 were encountered. In about one-half of the kittens, following the first significant fall in the leucocyte count there was a temporary or compensatory rise of from 2,000 to 3,000 cells per c.mm., followed by a fall, which from

TABLE I

Leucocyte Counts and Temperatures in a Group of Cases with Severe Illness

Cat	Died or killed	6th day		5th day		4th day		3rd day		2nd day		1st day		Day of death	
		White blood cells	Temperature	White blood cells	Temperature	White blood cells	Temperature	White blood cells	Temperature	White blood cells	Temperature	White blood cells	Temperature	White blood cells	Temperature
		per c.mm.	°F.	per c.mm.	°F	per c.mm.	°F.	per c.mm.	°F.	per c.mm.	°F.	per c.mm.	°F.	per c.mm.	°F.
1	D	—	—	31,300	—	—	101.2	9,900	103.0	9,500	101.6	1,500	102.6	—	—
2	D	—	—	—	—	21,300	—	—	101.2	5,200	103.8	200	104.0	—	—
5	D	25,400	102.4	9,950	102.2	5,450	102.4	6,150	102.0	3,300	104.2	1,250	104.4	200	104.4
6	K	19,100	101.8	18,000	102.6	8,850	102.6	9,150	103.0	11,500	102.5	{6,100 / 1,650}	{*104.2 / 103.8}	75	{*102.0 / 96.7}
7	K	—	—	—	—	14,000	102.0	12,050	101.8	4,150	102.8	600	104.9	0	100.8
11 C	D	—	—	—	—	—	—	15,300	103.6	1,050	105.0	450	103.0	—	—
11 D	D	—	—	—	—	—	—	—	—	—	—	350	103.6	—	—
11 E	D	14,100	102.0	17,900	101.8	14,900	103.2	5,900	102.7	10,700	102.4	200	105.0	—	98.6
11 F	K	—	—	19,800	103.0	21,300	103.7	7,750	104.4	9,300	103.5	550	{*104.4 / 104.4}	100	104.4
13	D	—	—	—	—	—	—	—	—	9,300	102.4	6,100	103.6	—	—
14	D	—	—	—	—	—	—	—	—	4,100	103.8	200	104.2	—	—
15	D	—	—	—	—	5,000	—	8,200	102.5	1,500	104.2	—	—	—	—
16	D	—	—	—	—	—	—	—	—	—	—	450	104.8	—	—
38	K	—	—	—	—	—	—	—	—	—	—	3,850	104.2	{*500 / 15}	104.6
Means ..		19,533	102.1	19,370	102.4	12,971	102.5	9,300	102.7	6,327	103.3	1,510	104.0	128	102.0

* Where two observations were recorded on the same day, the mean of the two has been used in computing the mean of the column.

then on was maintained except where manifestations of beginning recovery were noted (Tables II, IV, V).

In 5 instances of severe infection following inoculation daily differential white cell counts were made. In 2 of these animals the total granulocyte count decreased more rapidly than the total lymphocyte count, but in one cat the total lymphocyte count fell more rapidly than that of the granulocytes. In 2 others the normal relative proportion of granulocytes and lymphocytes remained more or

less constant up to the point where so few cells could be found on a smear that no significant count was possible.   Table IV contains an example of this latter group.

The large monocytes and basophile cells usually disappeared entirely from the circulation during the most severe phase of the leucopenia.

During the course of the illness in fatal cases no abnormally immature cells of any of the leucocyte series appeared in the circulation.

Because of the lack of any constant relative increase in lymphocytes and the marked reduction in all or nearly all types of leucocytes we do not regard the disease as an agranulocytosis as do Lawrence and Syverton (7).   Nevertheless because of the similarity of their findings in respect to the leucopenia with those reported here, we believe the disease which they have described to be probably the same as that which we have studied.

Hindle and Findlay (2) made leucocyte counts on a number of their cats suffering from distemper with enteric symptoms and pathology, and found them to show inconstant changes of no significance.   Accordingly it seems unlikely that this disease is the same as that with which we have been concerned.   Verge and Cristoforoni (1), Urbain (4), and Leasure and his coworkers (5) make no statement concerning any abnormality in the number of circulating leucocytes.

The changes in the blood cells of one cat which recovered after a serious infection are of considerable interest since they give indication of the involvement of the entire hematopoietic system.   Table II includes a record of the observations which were made during the course of the disease.

The disease reached its climax on the 6th day after inoculation, with a leucocyte count of 2,050, and a temperature of 104.7°F.   The animal ate nothing, appeared weak and was obviously dehydrated.   At this time a large number of abnormal, immature cells appeared in the blood, many of which could not be classified, although the greater part of them was probably of the lymphocyte series.   Following this, improvement was rapid and immature cells of both the red and the white cell series appeared.

Red Blood Cells.—Red blood cell counts were not made as routine, but those which have been carried out in 6 cats all indicated a decrease of approximately 1,000,000 cells per c.mm., which in the presence of terminal dehydration may represent a fall of 2,000,000 or 3,000,000 from the original normal.   The count in cat 4 which survived (Table II), had fallen just over one million cells at the height of the infection, but 7 days later, despite marked signs of regeneration of red cells, as indicated by the appearance of many immature erythrocytes, the count was over 2,000,000 less than the original.   The findings in these few animals together

with the evidence obtained from an examination of the bone marrow[3] of all which we have studied indicate clearly that there is a suppression of erythropoiesis, in addition to a leucopenia.

TABLE II

Clinical Record, Cat 4: Age: 6 months.   Inoculum: Unfiltered sterile suspension of lungs, liver and spleen from cat 1, made from organs which had been preserved in glycero-saline for 3 days.   Method of inoculation: Given subcutaneously.

Day	Temperature	Red blood cells	White blood cells	Polymorphonuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles	Myelocytes	Symptoms
	°F.	millions per c.mm.	per c.mm.	per cent	per cent	per cent	per cent	per cent	per cent	
Inoculated	102.5	7.35	9,100	75	9	6	8	2	0	—
1	102.0	—	11,650	82	9	5	2	2	0	Coryza
2	101.0	—	15,100	76	25	4	4	1	0	"
3	101.5	7.28	4,250	85	6	5	2	2	0	Coryza, anorexia
4	102.0	—	7,350	70	23	2	5	0	0	Coryza, anorexia, very quiet
5	103.0	—	2,650	40	52	0	8	0	0	No coryza, quiet, eating small amount
6	104.5	6.83	2,050	*	*	—	—	—	—	Dirty and sick, anorexia
7	104.0	—	6,000	*	*	—	—	—	—	Improved
8	101.8	—	23,500	61	27	0	0	0	12	Lively, eating
9	101.2	—	31,100	80	10	0	0	0	10	Eating better, almost normal
10	101.0	†	32,400	60	23	0	1	0	7	Normal
11	102.2	†	29,800	67	29	4	1	0	0	"
12	102.2	†	42,700	71	24	3	2	0	0	"
13	101.0	5.01	23,800	—	—	—	—	—	—	"
14	102.0	—	19,400	—	—	—	—	—	—	"
15	—	—	—	—	—	—	—	—	—	"
16	101.8	—	12,250	—	—	—	—	—	—	"

* Too many immature cells. Count impossible, lymphocytes predominating.
† Numerous polychromatophilic erythrocytes and a few normoblasts.

No systematic studies have been made as yet to determine whether the anemia may be due in part to destruction of adult erythrocytes,

[3] See page 335.

suggested by the finding of an unusually large number of phagocytic cells in the bone marrow, lymph nodes and spleen which have taken up many red blood cells, and a definite increase in icterus in the sera of two moribund animals compared to that of the sera of several normal cats. However no marked increase of pigment has been noted in cells of the reticulo-endothelial system. It is planned to determine the quantity of blood bilirubin, the percentage of hemoglobin and the reticulocyte count at intervals throughout the course of the disease.

Howell-Jolly bodies occur frequently in the normal cat's red blood cells, but in addition we have noted small, highly refractile bodies seen best in fresh unstained preparations, or in the counting chamber where the cells are laked by acetic acid. No marked change in the number of either of these cellular inclusions has been noted during the course of this disease.

Pathology[4]

Autopsies were performed on almost every cat dying from any cause, which came into our possession before death, and likewise on a number of cats obtained outside the laboratory, dying from various causes. 5 normal kittens of various ages were also autopsied and carefully studied. In practically every instance sections were made from material fixed in Zenker's solution and stained with hematoxylin and eosin or eosin and methylene blue. Tissue sections from at least two parts of the intestine, the marrow from the proximal end of the femur, the spleen and a large mesenteric lymph node located near the ileocecal junction were prepared from almost every autopsied cat, which included 49 cases of this disease. Occasionally a section of vertebral marrow was substituted for femoral marrow, but no essential difference was noted at any age. Besides these organs which we learned were specifically affected, a few infected cats were studied more or less completely. Thus, with the exception of the brain, only one of which was examined microscopically, the following organs were studied: salivary glands, esophagus, stomach, all levels of the intestinal tract, the lungs, heart, suprarenals, kidney, liver, bladder and pancreas.

It should be particularly emphasized that many of the infected animals coming to autopsy, and indeed those in which the most characteristic pathology was noted were either killed *in extremis* or in the earlier stages of the acute disease, and portions of the organs immediately placed in the fixative. Thus the intranuclear inclusion bodies, which appear as constant associates of this disease provided the material is taken under these conditions, are not the result of post mortem de-

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[4] We are grateful to Dr. Frederic Parker, Jr., Dr. Cecil K. Drinker and Dr. Henry Pinkerton for their kindness in examining many of our sections and offering valuable suggestions as to their interpretation.

generative changes in the cells. Indeed, when such changes have taken place, even to a relatively slight degree, it is often difficult or impossible to find inclusions in those sites in which they usually are easily manifest.

Bone Marrow.—This organ perhaps shows the most extensive damage. When the femur is carefully opened immediately after death, instead of the typical pencil like piece of fairly solid red marrow which extends almost the whole length of this bone in the kitten, one sees a small quantity of nearly fluid, or entirely fluid, bloody marrow. The marrow in the mid-shaft usually appears more normal than does that at the two extremities. This cannot be shelled out in one piece as in the normal cat, but may be scraped off with a dull, blunt instrument. It is unnecessary to decalcify the marrow from the cat's femur after fixation in Zenker's fluid.

On microscopic examination a very striking picture is presented in contrast to the normal marrow of the femur, which is not unlike human marrow (Figs. 3 and 4).

There are usually areas of very marked congestion, perhaps actual hemorrhage. Blood in these areas has replaced one-half or more of the usual cellular elements. Fibroblasts are conspicuous and a fine reticulum of tendrils with bulbous terminations is evident, with almost no cells in its mesh. Varying numbers of cellular elements remain, many of these in a degenerating condition with vacuolated cytoplasm and vesicular nucleus. Not infrequently large mononuclear cells, sometimes binucleated, contain eosinophilic intranuclear inclusions. Phagocytic cells are present with engulfed erythrocytes. Generally there is complete absence of all adult members of the white cell series but occasionally in cases where the leucocyte count failed to fall below 1,000 cells a few polymorphonuclear cells or sometimes fairly large numbers of eosinophile cells remain.

Practically complete aplasia of both the red and the white series of cellular elements is frequently found. In others a fair number of the red cell series may persist and in a third small group the reverse is seen, the members of the granulocytic series being more in evidence. Whenever blood counts have been made the condition revealed by the circulating blood is reflected in the marrow. When no white cells or only 100 or 200 cells per c.mm. have been present in the marrow, the most complete aplasia of the younger forms of the granulocytic series together with absence of all adult polymorphonuclear cells has been observed. Usually the most conspicuous cell persisting in the marrow is the megakaryocyte. But, in a few cases with decreased platelets and a prolonged bleeding time, the megakaryocytes of the marrow were reduced in number or were entirely absent.

The marrow of three cats deserves special mention:

Cat 5-9. This was one of 2 adult cats which we have seen die as a result of this

disease. No ante mortem blood studies were made, but the diagnosis was confirmed by post mortem findings. No mature polymorphonuclear cells could be found in the marrow but it exhibited an unusually high proportion of myelocytes and premyelocytes, quite similar to the marrow of agranulocytosis in the human being (9, 10). This possibly represents the difference between the reaction in the adult and the kitten, and may explain the greater apparent resistance of older cats. Severe depression of the marrow in this one case at least did not occur. It is quite likely that had this cat not given birth to kittens 2 weeks previously it would have recovered.

Cat 6-4: This cat, about 6 months of age, after definite exposure had a typical illness with the leucocyte count falling to 1,800 cells, at which time indication of recovery was suggested by a fall of body temperature and an increase in the leucocyte count. When the latter reached 2,800 the animal was killed. In the gross the marrow appeared normal but on section was seen not to contain the normal complement of cells. There were however numerous primitive cells in both the red and the granulocyte series, but practically no adult white cells were present.

Cat 1-56 (Table III): From the findings in this animal also, which was killed at the onset of recovery, there is much to suggest that the premature granulocytes of the bone marrow rapidly reestablish themselves once the turning point has been passed.

Lymph Node.—The large ileocecal lymph node has been carefully studied. This is usually larger than normal and soft. No evidence of inflammation or hemorrhage has ever been noted such as Leasure, Lienhardt and Taberner (5), also Hindle and Findlay (2) have described. The glands have all exhibited extensive edema and great widening of the sinusoids, but the pathology that appears to be more or less specific is the marked depletion of adult lymphocytes and injury to the cells forming the germinal centers. Inclusion bodies in all respects resembling those found in the intestinal mucosa have been noted in some of the cells of the germinal centers, which like those of the spleen reveal evidence of injury by their failure to take the stain well (Fig. 5). An additional conspicuous feature is the presence of many mononuclear phagocytes crowding the widened sinusoids and containing numerous erythrocytes.

Spleen.—The spleen is not abnormal in size, color or consistency in the gross. On section the lymphoid follicles resemble closely the lymph nodes just described except that the phagocytic cells are less in evidence. Inclusion bodies in variable numbers occur here also, mostly in the cells of the germinal centers (Fig. 6). Leasure, Lien-

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hardt and Taberner (5), as well as Hindle and Findlay (2) describe congestion of the spleen with hemorrhages in the lymph follicles, evidence of which we have failed to find in the spleens which we have examined, with one exception where there was marked congestion and perhaps hemorrhage about each lymph follicle.

Small Intestine.—In a few animals no abnormality could be noted in the intestine in the gross. Usually, however, some section of the small intestine was found to be slightly or moderately congested both on the serosal and mucosal surface. On the latter the areas of congestion appeared as pin-point petechiae. These probably represent the tips of congested villi. The mid-portion of the small intestines was most often involved, and next in frequency the lower ileum. Rarely the congestion extended the full length of the small intestine or included the cecum and proximal colon. In the intestine of 2 cats gross ulceration and necrosis and a pseudomembranous surface were noted. In 2 others a few tiny ulcers were seen in the ileum, but these cases were exceptional. The small intestine was always completely empty except for a small amount of yellowish, mucoid fluid.

On section at various levels one finding was almost constant wherever properly fixed intact mucous membrane remained in the crypts of the tubular glands. Many of the cells of the mucous membrane had large vesicular nuclei with the chromatin matter arranged along a thickened nuclear membrane, with the nucleolus staining a very dense purple and pushed to the periphery of the nucleus, and in the clear center of the latter were one or more comparatively large acidophilic inclusion bodies typical of the type associated with virus infections (Fig. 2). These bodies are in some instances concentrated into an almost homogeneous mass, but on close examination, even these can frequently be seen to be composed of closely packed, minute pink staining granules. In other cases these granules are scattered loosely throughout the center of the nucleus, but invariably they are surrounded by a clear halo which separates them from the nuclear membrane. A not infrequent finding is a very large nucleus, as large or larger than the original cell, with the nucleolus forming a wedge shaped band which divides the nucleus in two parts, each containing an inclusion body. The intranuclear bodies usually take a delicate pink stain with either hematoxylin and eosin or eosin and methylene blue in contrast to the more deeply staining bodies found in herpes and salivary gland diseases. No cytoplasmic inclusions have been found.

At certain levels of the intestine the mucous membrane seems to have disappeared (Fig. 1), especially on the lateral surfaces of the villi and in the crypts of the glands, but on closer study the membrane can often be distinguished as a

greatly flattened layer of cells lying against the connective tissue base. The cyto-plasm of the epithelial cells takes a light blue tint like that of the fibroblasts rather than the deep blue of the normal mucosal cell. In the portions where this flatten-ing of the epithelium occurs the gland lumen appears to have been distended, probably by fluid. Frequently the lumen contains a mass of necrotic gland cells. The flattened cells seldom contain inclusion bodies. In contrast with that which covers the lateral and basal portions of the villi the mucous membrane over the tips of these structures is more apt to be intact.

The capillaries of the intestinal villi and of the submucosa are often moderately congested and usually the submucosa is somewhat edematous. Inflammatory infiltration by polymorphonuclear leucocytes is strikingly absent but many mono-nuclear cells are present, principally plasma cells. As a rule no eosinophiles have been observed. Occasionally there are areas which suggest connective tissue proliferating in the villi. Again it should be clearly stated that when the autopsy is not done until several hours post mortem, the condition of the mucous membrane in the gland crypts is such that no inclusion bodies can be found. However, in most instances where the tissues were fixed immediately after death they could be easily demonstrated in fairly large numbers.

In the ileum the lymph follicles are hypertrophied but as in the lymph nodes and spleen the number of adult lymphocytes has been greatly reduced; and in their place an unidentified type of large mononuclear cell and edema fluid make up the sizeable mass representing the lymph nodes. In one case several intranuclear inclusions were seen in these large mononuclear cells.

Colon.—The lesions present in the proximal portion of the colon simulate rather closely the milder lesions in the small intestine. Fre-quently many inclusion bodies are present in the mucosal cells. The hypertrophy of the solitary lymphoid follicles is similar to that of the follicles in the ileum. As mentioned above, the proximal colon in a few cases has shown mild congestion in the gross, but no ulceration has ever been seen and the ileocecal valve has never shown any con-gestion or other abnormality. A congestion of the latter has been described by Wooldrige in his discussion of Dalling's paper (6) as a constant lesion in distemper of cats.

The whole pathological picture in the intestinal tract is remarkably constant, varying only in degree and not resembling any other in-testinal lesion that we have ever seen. Some of the descriptions of the milder forms of acute infectious enteritis of cats resemble our severe lesions in the gross, but nothing in these lesions of ours or in the symptoms they produce would justify the name of enteritis. The

remarkable thing about the lesion is the lack of inflammatory response in the presence of such extensive injury. Hindle and Findlay (2) describe gross and microscopic intestinal changes in distemper of the cat very similar to those which we have noted, but they failed to find after careful search any characteristic cytological appearances in the nucleus or cytoplasm. It will be remembered that they also found leucocyte counts to be of no value in recognizing the disease with which they worked. Leasure *et al.* (5) studied carefully the enteric pathology in infectious enteritis and apparently found no inclusion bodies. As a whole the intestinal pathology they describe was of a much more severe nature than ours, with marked ulceration and pseudomembranous lesions. They found the colon "usually devoid of feces or containing a blood-tinged mixture of feces and catarrhal exudate."

With the exception of the occasional presence of pneumonia, no significant changes have thus far been noted in the lungs, brain, kidney, bladder, suprarenal gland, salivary gland, heart, liver, pancreas or stomach. In the buccal cavity no glossitis or ulceration has been found, though looked for as routine since such lesions have been frequently mentioned in connection with acute infectious enteritis.

Etiology

In the demonstration of the nature of the etiological agent difficulty has been encountered because of its extreme infectivity. Apparently healthy animals brought to the laboratory and observed for a period of time varying from 8 to 18 days, either spontaneously developed characteristic symptoms or later proved refractory to the inoculation of material from sick animals or to contact exposure to cats suffering from the infection. To our minds this lack of response on the part of certain cats is most readily explained on the assumption that the animals possessed a natural immunity which, because they were nearly all under 6 months of age, was most likely in the form of a passively transferred congenital resistance. All attempts up to the present time to devise satisfactory techniques for isolating the animals under the conditions available in Boston, where the disease is prevalent, have failed. However, a single experiment carried out on a farm in Connecticut has yielded evidence which indicates that the disease

can be transmitted by means of filtered material obtained from an infected animal.[5]

[5] A second experiment has since been carried out which affords further evidence in support of the conclusion that the disease is due to a filterable agent.

Three kittens from a rural district in Pennsylvania were isolated for 27 days in quarters at a distance from the laboratory. During this period no signs suggestive of the specific infection were noted. The probable susceptibility of these animals to the disease was indicated by the fact that 20 other kittens in the same shipment which were placed in an enclosure previously occupied by infected cats, all died with characteristic findings after 8 to 15 days.

Two of the isolated kittens were given intraperitoneally 5 cc. of a filtrate which was prepared in the following manner. The spleen of a cat dying of the infection was thawed after being maintained in the frozen state at $-10°C$. for 16 days. An 8 per cent emulsion of the organ was then prepared in the manner already described in the text. Filtration of the centrifuged emulsion was effected by means of a Berkefeld V filter at 20 cm. of Hg negative pressure. This filtrate proved sterile after 8 days incubation in suitable media both under aerobic and anaerobic conditions.

The third kitten which served as a control received intraperitoneally 5 cc. of a mixture of equal parts of infusion broth and saline. It was then placed in a cage at a distance of 25 feet from the 2 inoculated animals.

On the 6th day, one of the inoculated kittens died. During the last 2 days of life it presented the typical signs and the leucocytic count rapidly fell to 0. Post mortem findings in the gross and the histological changes proved the presence of the disease. The second animal which received the filtrate recovered after an illness in which the leucocytic count reached 3,925 on the 7th day following inoculation, when the temperature was 105°F. During recovery, blood studies revealed indication of marked and rapid regenerative changes, especially in the leucocytes similar to those observed in the blood of cat 4 (see text).

The control animal remained well for 18 days. Then it was inoculated intraperitoneally with a Berkefeld filtrate of a 10 per cent suspension of lymph node and spleen from 2 infected animals. The lymph node had been kept in the frozen state for 8 days and the spleen for 34 days. Because of difficulty in filtering this suspension, much of it was lost. Therefore, to insure adequate exposure to the virus, a few drops of the unfiltered suspension were also instilled intranasally without anesthesia. After an incubation period of 5 days, the kitten became sick, developed a severe leucopenia and died on the 7th day following inoculation. Just before death, only 200 leucocytes were found in the blood. The post mortem findings in the gross were typical of the infection. Thus we believe that there can be no doubt concerning the cause of death, although microscopic sections of the viscera have as yet not been examined. The fact that the control kitten proved

Four young animals about 4 months old were obtained locally and brought to the farm where they were observed for 3 weeks, during which period they remained free of any obvious signs of disease. The kittens were derived from different sources and were not litter mates. 2 of the cats were then selected at random and removed to a building about 300 yards distant from their original quarters, where the remaining 2 animals were confined during the experimental period. The latter served to control further the possible occurrence of spontaneous disease which might have arisen as a result of infection which had not yet become apparent among the stock before its separation. These control animals were fed by the same attendant who had previously cared for all the cats during the preliminary period of observation and who subsequently had no contact with the inoculated animals. Moreover we refrained from examining the control animals except from a distance, relying upon the report of the attendant and ocular evidence for the absence of any sign of illness.

The 2 experimental animals, Nos. 1-56 and 1-57, were each inoculated intraperitoneally with 3 cc. of a Berkefeld N filtrate of the emulsion of a spleen preserved for 34 days at −10°C. which had been removed from a cat killed in the acute stage of the disease contracted spontaneously. Both the clinical and pathological evidence obtained in the case of the animal yielding this material revealed the presence of the typical disease. The emulsion was prepared in the laboratory at Boston in the following manner: 3 gm. of splenic tissue together with pyrex glass capillary tubing were ground in a mortar with the gradual addition of 30 cc. of a mixture containing equal parts of Tyrode's solution and infusion broth. The emulsion was centrifuged for 50 minutes at 2000 R.P.M. The supernatant fluid was then run through a Berkefeld N candle at a negative pressure of 60 cm. Hg. 5 drops of the filtrate were inoculated into each of two tubes of cooked meat infusion broth and two tubes of rabbit blood broth. Blood agar plates were likewise inoculated with 2 drops of the filtrate. No growth of bacteria occurred in any of these media after 8 days at 37°C. The bulk of the filtrate was placed in sterile sealed containers and kept at 0°C. in a vacuum jar during transportation from the laboratory in Boston to Waterford, Connecticut. On arrival the containers were put in the ice box until the next day when their contents were injected into the cats 1-56 and 1-57. Total white blood cell counts and erythrocyte counts were carried out immediately prior to inoculation and daily thereafter at approximately the same hour, which was always before the morning feeding. The temperature at this time was likewise recorded. The data thus obtained are presented in Table III.

susceptible to subsequent inoculation affords additional evidence that the disease produced in the 2 experimental animals resulted from inoculation and not from natural infection through materials inadvertently introduced into their quarters, either by the attendant or by some other agency.

Most significant is the evidence for the occurrence of a definite leucopenia in both cats which had received the filtrate. This was extremely marked in 1-56, while less severe but, we believe, definite in 1-57.[6] The behavior of cat 1-56 conforms with our earlier findings in that the decrease in the number of leucocytes

TABLE III

Clinical Record, Cats 1-56 and 1-57: Age: 4 months. Inoculum: Suspension of spleen of cat 1-17, frozen at −10°C. for 34 days and passed through a Berkefeld N filter. Filtrate sterile. Method of inoculation: Intraperitoneal, 3 cc.

Day	Cat 1-56				Cat 1-57			
	Temperature	Red blood cells	White blood cells	Symptoms	Temperature	Red blood cells	White blood cells	Symptoms
	°F.	millions per c.mm.	per c.mm.		°F.	millions per c.mm.	per c.mm.	
Inoculated	102.0	6.17	15,800	Normal	102.5	6.70	20,400	Normal
1	101.5	6.99	13,000	"	102.4	5.96	13,800	"
2	102.2	7.35	10,200	"	102.0	6.20	7,400	"
3	101.3	6.97	10,000	"	101.3	5.87	7,800	"
4	102.8	6.71	6,800	"	102.5	5.23	5,700	"
5	102.4	5.70	5,200	"	101.4	—	8,400	"
6	105.3	6.10	1,200	Anorexia, vomiting, quiet and obviously sick	101.6	4.68	11,000	"
7, a.m.	101.0	5.90	1,000	Anorexia, quiet, weak, very sick	101.6	6.22	11,400	"
7, p.m.	105.2	—	1,600	Vomiting, drinking water, weak and very sick	—	—	—	—
8	101.2	5.25	8,800	Improving, respiratory infection. Killed	101.4	7.33	10,600	Normal, observed for 2 wks.

precedes the appearance of recognizable illness manifested by the refusal of food, vomiting, increase in temperature and marked weakness. None of the typical clinical manifestations of acute and severe illness accompanied the drop in the leucocytic count of cat 1-57. Here the disease if present was evidently extremely mild.

Although less marked the red blood cell counts in both animals indicated a

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[6] For discussion of range of variation in number of leucocytes see page 330.

definite decrease in the numbers of erythrocytes during the course of the disease. This likewise is in keeping with their behavior in animals previously studied.

Cat 1-56 which in all respects underwent a typical course and was so ill that death appeared imminent during a period of 48 hours, was killed and autopsied at the time when the increasing numbers of leucocytes and the general improvement in the animal's condition denoted the inception of recovery. In the gross no definite pathological lesions were evident. The small intestine throughout its entire length was empty except for a slight amount of bile-stained viscous fluid, while a scanty amount of formed feces was present in the colon. This condition of the intestinal contents, it will be recalled, is characteristic of the spontaneous disease. In the lower ileum the mucosa was slightly roughened and pinkish in certain areas. The femoral bone marrow unlike that found in the acute stage or when the animal has died as a result of the infection was not abnormally soft or hemorrhagic. It was however definitely paler than in the normal animal.

Histological examination of sections of the intestine which had been placed in Zenker's fluid within 10 minutes after death showed that the greater portion of the mucosa was normal. However between groups of glands and villi bearing intact epithelial cells, other glands were interspersed either singly or in groups which were lined with severely damaged epithelium. Certain of the necrotic cells lay in the lumina of the glands; others, in various stages of degeneration, remained *in situ*. In places the basal membrane was covered by a small number of flattened cells. After prolonged examination four typical intranuclear inclusion bodies were observed in the epithelial cells of the glandular acini. There was no evidence of any inflammatory reaction. The areas of normal mucosa showed no evidence of autolysis or post mortem degeneration. The lesions which have been described were most conspicuous in the ileum, but similar areas of injured mucosal tissue were noted in the jejunum.

The bone marrow contained an abnormally large number of primitive cells belonging to the white blood cell series, most of which appeared to be premyelocytes. A minority of these cells were eosinophiles with bilobate nuclei. A very striking feature was the presence of numerous mitotic figures amounting to at least one per oil immersion field. Exceedingly few representatives of the erythrocytic series of cells were seen. In respect to this series one could regard the histological findings as indicating an aplastic marrow. No inclusion bodies were found.

In general the situation in the marrow conforms entirely with that which might be expected during recovery from the severe injury to this organ which has been found to take place during the acute stage.

The lymphoid tissue (mesenteric lymph node) appeared definitely abnormal in three respects: (a) The germinal centers contained practically no lymphocytes, but were replete with reticulum cells which appeared to be for the greater part completely inactive. No mitotic figures were seen in any of the centers except

in one part of the node where regeneration seemed to be commencing and some medium sized lymphocytes were beginning to appear in the germinal center. (*b*) The density of distribution of lymphocytes both around the germ centers and in the medullary cords was much less than normal. Many of the cords had disappeared. At the same time it was noteworthy that those lymphocytes which were present did not appear to be abnormal in any respect. (*c*) Many of the cortical sinuses were almost empty. The medullary sinuses, on the other hand, were full of proliferated reticulo-endothelial cells. No inclusion bodies were found in this organ.

Here again there is definite indication of the type of damage which we have found to be typical of the disease as it affects this organ. But the regenerative processes of the lymphoid cells, unlike those of the granulocytic series, has at this early stage of recovery scarcely begun.

In conclusion, then, the post mortem findings support and supplement the conclusion which may be drawn from the evidence presented by the clinical course, that the disease produced in this animal following the injection of a filtrate free of bacteria was identical with the natural infection as it occurred in the cat from which the material used in the preparation of the filtrate was derived.

We cannot unreservedly assert that the other animal inoculated with the filtrate developed the disease. It is nevertheless highly probable that a mild infection occurred expressed only by a moderate leucopenia and a fall in the erythrocyte count. That a leucopenia actually was present is indicated by the fact that as we have previously stated we have never observed the total white blood cells in normal animals to fall below 8,900 per c.mm. except in one instance which may well have been due to error in technique. It is not surprising that both animals failed to respond in a similar manner since we have had much evidence which shows that resistant cats are not infrequently encountered.

This experiment in addition to exhibiting the filterable nature of the agent shows that the period of incubation as measured by the time elapsing between infection and the onset of manifest signs of illness is about 6 days. The leucopenia, however, begins at least 2 days earlier.

In the light of the information derived from the foregoing experiment

we believe that the results of two of the experiments previously carried out and designed to test the infectivity of bacteria-free filtrates of the emulsion of various organs obtained from typical cases of the disease are of confirmatory value in indicating the filterable nature of the infectious agent.

TABLE IV

Clinical Record, Cat 11-E: Age: 4 months. Inoculum: 2 cc. of nasal washings from cat 5, taken in normal saline and frozen at −10°C. for 45 days, thawed and passed through a Berkefeld V filter, proved sterile by culture. Method of inoculation: Given intranasally under light ether anesthesia.

Day	Temperature	White blood cells	Differential cell count					Platelets	Symptoms
			Polymorphonuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles		
	°F.	per c.mm.	per cent	per cent	per cent	per cent	per cent		
Inoculated	102.2	18,600	72	21	2	5	0	Normal	Normal
1	102.6	17,800	54	37	3	5	1	"	"
2	102.0	14,100	61	20	1	18	0	"	"
3	101.8	17,900	77	13	1	9	0	"	"
4	103.2	14,900	55	26	4	15	0	Decreased	"
5	102.7	5,900	80	16	1	3	0	"	"
6	102.4	10,700	74	22	0	4	0	Normal	Anorexia, quiet in p.m.
7	105.0	200	*—	—	—	—	—	—	Anorexia, weak, languid, vomiting
8	98.0	—	*—	—	—	—	—	—	Vomiting. Died in convulsions

* Not sufficient number of white cells found on smear to make satisfactory count.

Cat 11-E: The details of the inoculation and the clinical course with laboratory studies are presented in Table IV. This animal was brought directly to the laboratory and inoculated. It had been reared on a farm where no cat had been known to be ill previously. It died 8 days after inoculation, of an illness in every

respect typical, and the diagnosis was confirmed by all the pathological criteria we have mentioned above.

Cat 11-F: The data regarding inoculation and the clinical course in this animal is presented in Table V. This kitten came from the same source as the one described previously, but was kept overnight, in a laboratory room not used for cats for a 6 week interval, before inoculation. It was killed *in extremis* 5 days after inoculation and the diagnosis verified by study of the routine sections.

<div align="center">TABLE V</div>

Clinical Record, Cat 11-F: Age: 4 months. Inoculum: suspension of feces and intestinal mucosa of cat 11-E, passed through a Berkefeld N filter, proved sterile by culture. Method of inoculation: Given intraperitoneally without anesthesia.

Day	Temperature	White blood cells	Differential cell count					Platelets	Symptoms
			Polymorphonuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles		
	°*F.*	*per c.mm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Inoculated	103.0	19,800	—	—	—	—	—	Normal	Normal
1	103.7	21,300	80	13	2	5	0	"	"
2	104.4	7,750	87	6	0	7	0	"	"
3	103.5	9,300	68	24	0	8	0	"	Slight anorexia. Less active, vomited
4	104.4	5,050	85	10	0	5	0	"	Anorexia
5	104.4	100	—	—	—	—	—	—	Very weak, vomiting. Killed

An analysis of the many spontaneous cases which have occurred in animals brought to the laboratory indicates that the usual interval between probable exposure and death in spontaneous infections is from 10 to 15 days. The relative brevity of the period between inoculation and death in these 2 cats, 8 and 5 days respectively, which is of the same order as that noted in the Connecticut experiment, suggests that the disease resulted from inoculation of sterile filtered material.

The findings in the bacteriological study of the blood and viscera

in a considerable number of characteristic cases supports the direct evidence already presented which leads us to regard the infection as due to a filterable virus.

In 30 cases of the typical disease blood cultures were carried out using both blood agar and rabbit blood infusion broth as the media. Under aerobic conditions at 37°C. in 10 only did bacterial growth result. Of these 10 positive cultures, 4 were found to be organisms belonging to the genus Pasteurella; one a mixture of *Bacillus mesentericus* and *Staphylococcus albus*, 2 Gram-negative rods probably

TABLE VI

Inoculation of Other Animal Species

Animal	Number employed	Route of inoculation	Result
Rabbit	1 adult	Subcutaneous	Negative
"	1 adult	Scarified cornea	"
"	1 young	Intraperitoneal	"
Guinea pig	2	Intramuscular and intranasal	"
" "	2	Subcutaneous	"
" "	1	Intraperitoneal	"
" "	2	Intraperitoneal and intratesticular	"
Ferret	1	Intraperitoneal	"
"	1	Intraperitoneal and intranasal	"
Mouse	10	Intracerebral (3 experiments)	"
"	18	6 serial passages of brain material	"
"	4	Intracerebral and intranasal	"
"	4	Subcutaneous	"

B. coli, a hemolytic streptococcus, and the 2 remaining cultures were not identified. Several of these different species were inoculated into healthy cats. In no instance did they produce any condition resembling the original disease.

Cultivation of portions of various viscera of cats dying with the infection also yielded no growth or a diversity of bacterial species. Moreover anaerobic cultivation in certain instances of both blood and viscera gave results similar to those obtained by the aerobic method. With the exception of the lungs, in which occasionally pneumonia was present and one spleen, no positive cultures were obtained from any organ in the absence of a bacteriemia.

Considering the diversity of species encountered, these organisms in all probability represented secondary invaders which gained a foothold due to the almost complete absence of mobile phagocytic cells.

Insusceptibility of Other Species

As yet we have failed to produce pathological changes or any other indications of infection by the inoculation of tissues from infected cats into several of the common laboratory animals. Table VI contains a summary of these experiments.

Thus far the chorio-allantoic membrane of the developing chick has likewise failed to reveal any recognizable injury following contact with the same materials.

The pathogenic agent thus appears to be closely adapted to the natural host and in this respect is likewise comparable to certain of the known filterable viruses.

DISCUSSION

The infectious disease which has been described presents a most unusual group of pathological conditions, seeming entirely unrelated. In every kitten killed in the acute stage or dying of the disease a loss of circulating leucocytes of all kinds occurred, accompanied by marked aplasia of the bone marrow, aplasia and necrosis of lymphoid follicles, congestion of the intestines and necrosis of the mucosa of the intestinal glands. Usually when erythrocyte counts were carried out an anemia was noted and in exceptional cases the platelets in the circulating blood as well as the megakaryocytes of the marrow were markedly diminished in number. Characteristic intranuclear inclusion bodies were found in certain cells of the organs affected. Both the inclusion bodies and the other pathological changes apparently occurred in two distinct types of tissue, those of mesodermal and of entodermal origin. Although the cells containing inclusion bodies in the bone marrow, lymph nodes and spleen could not be absolutely identified, they were probably of mesodermal origin as are all the important functional elements of the bone marrow and the lymph glands. In the intestine the mucosal cells of entodermal origin are the ones principally affected.

Since the leucopenia developed prior to other signs in most cases, the lesion in the blood-forming organs probably represented the primary and most significant injury as far as the life of the animal was concerned. Although constantly present the lesions of the intestinal

mucosa varied greatly in severity and extent. Accordingly it is difficult to estimate their importance in so far as they might have contributed to the outcome.

The actual cause of death in our animals has not been determined. A complete absence of leucocytes should, of course, invariably lead to eventual secondary infection and septicemia, but many of the cats have died so rapidly that opportunity for secondary infection seems not to have been afforded.

The question as to whether or not this infection is to be identified with cat distemper or feline infections described by the various authors which have been cited is difficult to answer. The milder intestinal injury which we have noted, the failure on the part of other observers to describe the inclusion bodies which in our disease are conspicuous, the difference in the pathological changes in the lymph nodes, and the fact that one report specifically stated that no significant changes occurred in the number of leucocytes in the blood, strongly suggest that there exist more than one epizootic of cats which affect the gastro-intestinal tract.

Examples of diseases in which a filterable virus has been shown to be the etiological agent and in which the leucocytes on the one hand and the erythrocytes on the other are characteristically affected may be found in the leukemia of fowls and equine anemia. But in so far as we are aware with the exception of the malady recently reported by Lawrence and Syverton and which we believe to be identical with that which we have studied, no disease of virus origin has been described in which a severe fulminating leucopenia of both granulocytic and lymphocytic series forms a characteristic feature. Moreover we do not know of any other disease accompanied by constant and marked enteric pathology with the possible exception of the group of feline distempers referred to above, in which a virus may be causally related. In this connection, however, the observations of Slanetz and Smetana (11) should be recalled, who have described intranuclear as well as intracytoplasmic inclusion bodies in the epithelium of the intestinal tract of ferrets dying from an epizootic disease, shown by them to be due to a filterable agent. But they fail to mention any other indication of injury to the intestinal mucosa. In canine distemper DeMon-

breun (12) also observed inclusions in the intestinal epithelium of infected animals, but with little evidence of accompanying damage to the mucosa.

The mode of natural transmission has not been established, but it would seem most probable to take place by direct contact with the discharge of infected animals. The possibility, however, that an insect vector may occasionally be involved cannot be disregarded.

Our experience has indicated that the natural disease is highly infectious and that the agent either remains viable for considerable periods of time outside the body or possibly in that of an intermediate host such as the flea. Satisfactorily controlled experiments have therefore proved extremely difficult to carry out. To analyze the many interesting aspects of the disease suggested by our observations, more convenient techniques for securing rigid isolation must be devised.

SUMMARY

An acute, highly fatal epizootic disease of cats is described, which can be recognized by a fulminating and extreme leucopenia involving all types of white blood cells, aplasia of the bone marrow, including both the granulocytic and the erythrocytic series and occasionally the megakaryocytes, aplasia of lymphoid tissue, and characteristic intranuclear inclusion bodies in the cells of the intestinal mucosa and in certain cells of the spleen, lymph nodes and bone marrow.

The infection has been induced in healthy cats by means of bacteria-free filtrates of emulsions of the spleen of infected animals. Collateral evidence supports the conclusion that the disease is due to a virus. The pathogenicity of the infectious agent has proved thus far to be strictly limited to the natural host.

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EXPLANATION OF PLATE 18

FIG. 1. Low power magnification of the mucosa in the ileum of cat 11-F, killed *in extremis*. Less than half of the tubular glands are lined with normally staining epithelium. The cellular infiltration consists almost entirely of plasma cells. Eosin-methylene blue. × 80.

FIG. 2. High power magnification of the fundus of a typically affected intestinal gland. In the lumen are necrotic cells and in one epithelial cell is a characteristic eosinophilic intranuclear inclusion body. Hematoxylin and eosin. × 950.

FIG. 3. Low power magnification of marrow from femur of kitten, 3 months of age, which died from a spontaneously acquired infection. There is marked aplasia of normal cellular elements. Eosin-methylene blue. × 100.

FIG. 4. Low power magnification of marrow from femur of a normal kitten, 3 months of age. For comparison with Fig. 1. Eosin-methylene blue. × 100.

FIG. 5. The central area of a degenerated lymph follicle in a mesenteric lymph node. In the center is a mononuclear cell containing an eosinophilic intranuclear inclusion body (arrow). Very few adult lymphocytes remain. Hematoxylin and eosin. × 950.

FIG. 6. Section at the edge of a splenic lymphoid follicle. At the center is a cell with an eosinophilic intranuclear inclusion body. Hematoxylin and eosin. × 950.
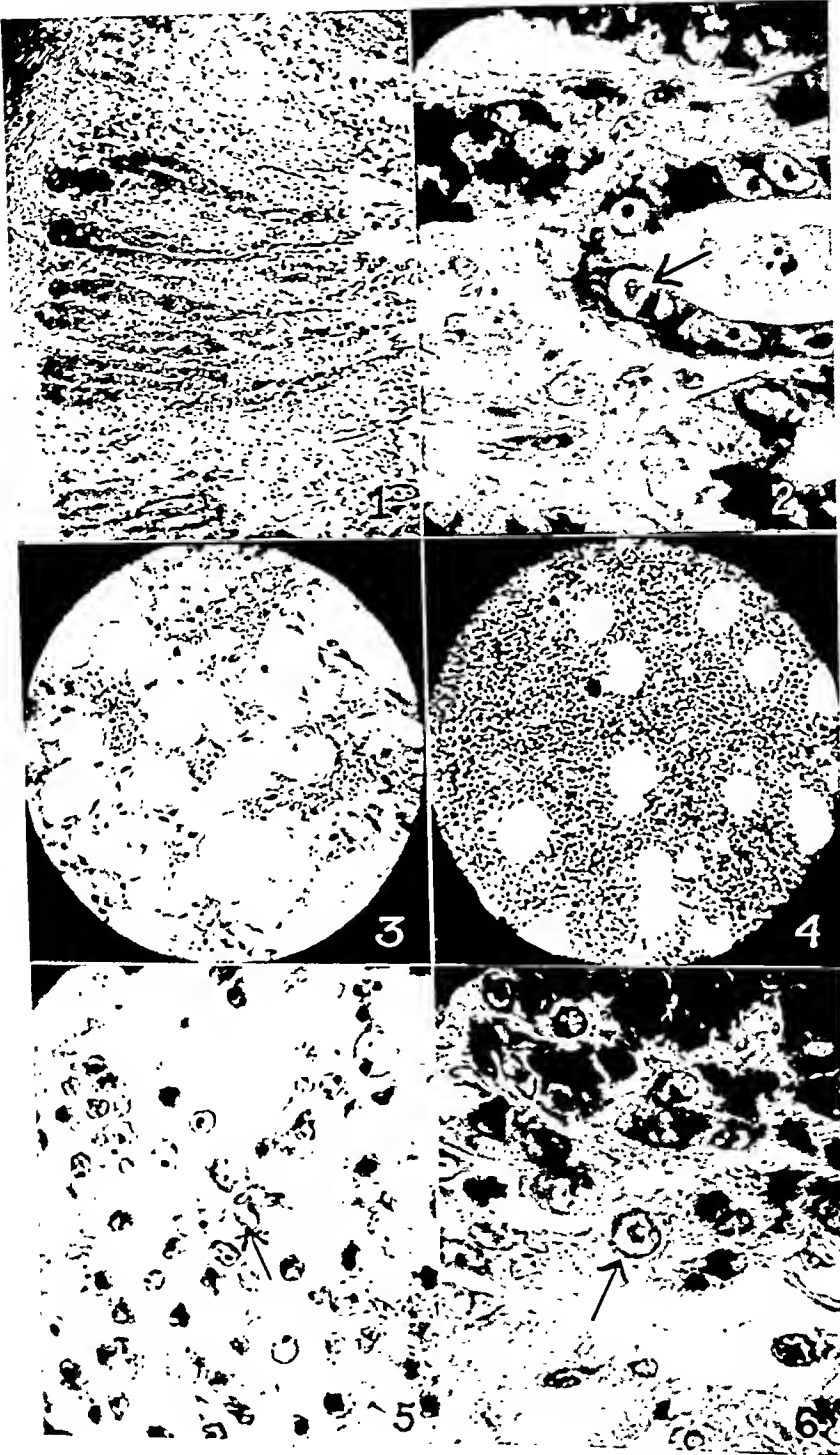
STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

I. Immunity to Experimental Pneumococcal Infection with an Antigen Containing Cellobiuronic Acid

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The results of experimental studies on artificial antigens containing the azobenzyl glycosides of glucuronic, galacturonic, and cellobiuronic acids indicate that the hexose uronic and aldobionic acids have an important function in determining the immunological characteristics of certain of the specific polysaccharides of encapsulated microorganisms (1). Azoproteins containing these uronic acids have the property of precipitating in high dilutions in antipneumococcal sera of various types, whereas antigens containing the azobenzyl glycosides of the corresponding aldoses show little or no serological activity. That the hexose uronic acid antigens actually combine with and precipitate the type specific polysaccharide antibodies has been demonstrated in a number of ways. It is apparent, therefore, that the artificial hexose uronic acid antigens possess certain of the serological characteristics of the immunologically active pneumococcus polysaccharides themselves. Despite this similarity, however, it has thus far been impossible to induce antibacterial immunity by injecting animals with the glucuronic or galacturonic acid antigens. Attempts to induce immunity to Type III pneumococcal infections in mice, rabbits, goats, and horses with an azoprotein containing glucuronic acid have all been unsuccessful. The reason probably resides in the fact that glucuronic acid alone does not approximate closely enough the chemical structure of the more complex building stone of the Type III pneumococcus polysaccharide, cellobiuronic acid (2).

Recently it has been shown that an azoprotein containing cellobiuronic acid simulates much more closely the serological character-

istics of the capsular polysaccharide of Type III Pneumococcus than does one containing glucuronic acid (1 c). These observations have led to the belief that in order to induce Type III antipneumococcal immunity in experimental animals with an artificial antigen, it is not necessarily essential to have as the immuno-specific group the long chained type specific polysaccharide (3), but that the building stone from which it is constituted, namely the aldobionic acid, should suffice. In accepting this point of view it must be borne in mind that the polysaccharides, biologically active and otherwise, are simpler entities than are the antigenic proteins. The carbohydrates may in general be regarded as constituted from a fundamental pattern unit of one or more simple saccharides combined in glycosidic union to form a long chained macromolecule. Whether in artificially compounded antigens the integrity of the bacterial polysaccharide molecule is essential for the expression of type specificity and the capacity to induce antibacterial immunity, or whether the pattern unit, which in the case of the Type III Pneumococcus is cellobiuronic acid, will suffice, is the subject of the present investigation.

Two artificial azoprotein antigens have therefore been prepared, one containing the azobenzyl glycoside of the disaccharide cellobiose, the other the corresponding glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus specific polysaccharide. The structural relationship of these two glycosides is represented by the following formulae:
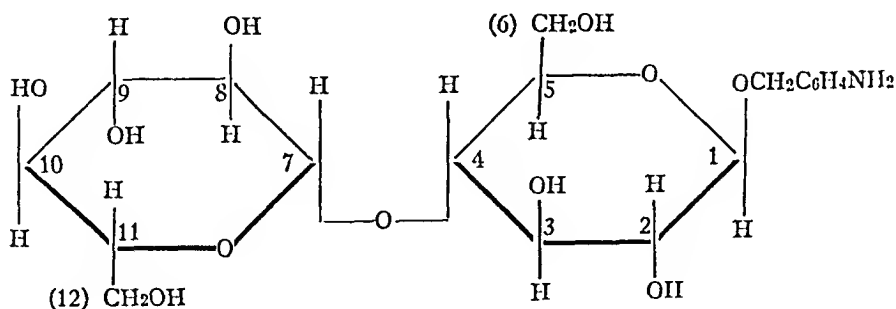
p-aminobenzyl *β*-cellobioside
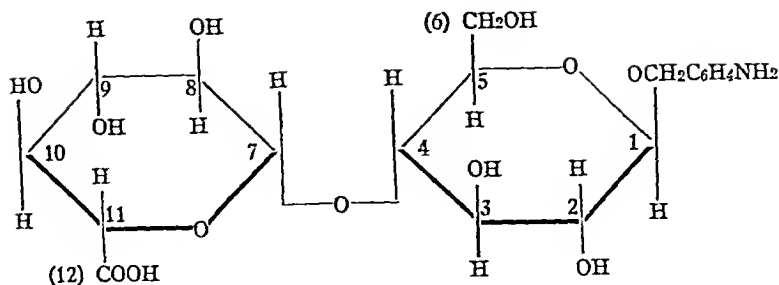
p-aminobenzyl *β*-cellobiuronide

These two substances differ only in the grouping occupying the 12th position which in the cellobiose is a primary alcohol grouping (CH_2OH) and in the cellobiuronide a carboxyl group (COOH). From the following account it will be seen that this slight difference in chemical constitution confers upon each antigen vastly different immunological properties.

EXPERIMENTAL

Methods

Immunization.—Two groups of rabbits weighing from 2 to 2.5 kilos were immunized respectively by the intravenous injection of 1 cc. of a sterile 0.5 per cent solution of the cellobiose and cellobiuronic acid antigens. The latter were prepared by combining the diazotized derivatives of the *p*-aminobenzyl glycosides of cellobiose and cellobiuronic acid to horse serum globulin as previously described (1 *c*). The animals received six daily doses of antigen and after a rest period of 7 days, the course of injections was repeated a second time. When necessary a third course of immunization was given. 7 days after the last injection the animals were bled from the ear and the sterile serum kept without preservative. In the tables the immunizing antigens will be referred to as cellobiose-globulin and cellobiuronic acid-globulin.

Technique of Immunity Reactions.—In the precipitin reactions, test antigens were prepared by combining the diazotized glycosides to the protein of chicken serum in order to avoid protein cross-reactions. In the agglutination reactions the antiserum to be tested was diluted with the appropriate quantity of sterile saline and an equal quantity of freshly prepared suspensions of heat-killed (70°) pneumococci of the specific type indicated in the protocols was added. All tubes were incubated at 37° for 2 hours and readings made after 24 hours in the ice chest.

The protection tests were done by the conventional technique; mice were injected intraperitoneally with 0.2 cc. of immune serum together with graded amounts of virulent cultures of pneumococci. The dilutions were so made that

in all instances the total volume injected was 1 cc. Only those antisera having
maximal precipitin titre for the homologous test antigen were used in mice, since
antisera of lower titres failed to show appreciable protective action against viru
lent pneumococci. Following the course of intravenous injections the rabbits
were tested for active immunity by the intradermal method of Goodner (4), using
a culture of a rabbit virulent strain of Type III Pneumococcus, 0.001 cc. of which
killed normal animals within 48 hours. The extent and character of the lesions,
as well as the temperature of the animals, were recorded daily. All animals were
observed for a period of 18 days before terminating the experiments.

RESULTS

Precipitins.—The immunization of the rabbits with the azoprotein
antigens containing cellobiose and cellobiuronic acid was followed by
means of the precipitin test. Two antisera obtained from each of two
groups of rabbits which had received the cellobiuronic acid antigen
were chosen for further investigation. Two cellobiose antisera like-
wise obtained from two groups of each of three rabbits were used in
the immunological studies. All of these antisera yielded a marked
precipitate with high dilutions of homologous test antigens.

Neufeld "Quellung" Reactions.—Using the standard technique for the
Neufeld reaction it was found that a young actively growing culture of
Type III Pneumococcus when mixed with cellobiuronic acid antiserum
showed a typical and unmistakable swelling of the capsule indistin-
guishable from the Neufeld reaction produced by Type III anti-
pneumococcus rabbit serum. The specificity of this reaction is the
more striking since antisera to the cellobiose antigen failed to produce
swelling of the capsule of Type III pneumococci. No swelling of the
capsules of Types II and VIII pneumococci could be observed when
the respective organisms were tested with cellobiose or cellobiuronic
acid antisera. This point will be discussed further in the section
dealing with the protective action of these sera.

Agglutinins.—The sera of rabbits injected with the cellobiose and
cellobiuronic acid antigens were tested for agglutinins with heated
suspensions of Types II, III, and VIII pneumococci. The results of
typical experiments are given in Table I. From the results given in
Table I it can be seen that the sera of rabbits immunized with the
cellobiose antigen failed to agglutinate, in the range of dilutions used,
any of the types of pneumococci tested. On the other hand, the

ellobiuronic acid antisera in high dilutions agglutinated specifically
[ype III pneumococci but not the organisms of Types II and VIII.
These experiments were carefully controlled in that the serum of the
;ame animals obtained before immunization was similarly tested and
n each instance found to be wholly devoid of specific antibodies for
Type III pneumococci.

From the results of these experiments it can be concluded that the
antisera of rabbits immunized with the artificial cellobiuronic acid
antigen contain antibodies which cause swelling of the capsules and
agglutination of Type III pneumococci, whereas the cellobiose antisera
show neither of these properties. It is apparent, therefore, that the

TABLE I

Agglutination of Types II, III, and VIII Pneumococci in Cellobiose and
Cellobiuronic Acid Antisera

Antiserum prepared by immunization with	Pneumo- coccus Types	Final dilution of serum						
		1:5	1:10	1:20	1:40	1:80	1:160	1:320
Cellobiose-globulin	II	0	0	0	0	—	—	—
	III	±	0	0	0	—	—	—
	VIII	0	0	0	0	—	—	—
Cellobiuronic acid-glob- ulin	II	0	0	0	0	—	—	—
	III	++	++	+++	+++	++	+	±
	VIII	±	0	0	0	0	0	0

conversion of the primary alcohol group on the 12th carbon atom of
cellobiose to the carboxyl group confers upon the cellobiuronic acid a
new and important immuno-chemical function.

Protective Antibodies: A. Cellobiuronic Acid Antiserum.—It has
been found that the capsular polysaccharides of Types III and VIII
pneumococci both contain cellobiuronic acid as an important con-
stituent of the molecule (5). In order to determine whether sera of
rabbits immunized with the artificial cellobiuronic acid antigen will
confer passive immunity on mice against infection with these types of
pneumococci, protection tests were performed by the technique de-
scribed. For purposes of comparison tests against infection with Type
I pneumococci were included in this experiment as well. Since the

capsular polysaccharide of Type I Pneumococcus bears no structural similarity to that of Types III or VIII, one would not anticipate any protective action of the cellobiuronic acid antiserum against infection with organisms of Type I.

The results of the protection experiments given in Table II show that the serum of a rabbit immunized with the artificial cellobiuronic acid antigen is effective in protecting mice against infection with 10,000 and 1000 minimal lethal doses of Types III and VIII pneumo-

TABLE II

Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice*

Amount of culture	Pneumococcus							
	Type I		Type III			Type VIII		
cc.								
10^{-3}	—	—	D 48	D 48	S	—	—	—
10^{-4}	—	—	S	S	S	D 44	D 72	S
10^{-5}	D 40	D 40	S	S	S	S	S	S
10^{-6}	D 40	D 48	S	S	S	S	S	S
Virulence controls† (no serum)								
10^{-6}	D 40		D 32			D 28		
10^{-7}	D 48		D 48			D 28		
10^{-8}	D 48		D 48			S		

* The serum of a rabbit immunized with the first preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† The number of colonies developing in blood agar, seeded with the 10^{-7} and 10^{-8} dilutions were in all instances counted (Tables II to IV).

cocci respectively. As was anticipated, the anticellobiuronic acid serum failed to protect against infection with virulent Type I pneumococci.

These experiments proved so striking that it was thought advisable to repeat them. Consequently an entirely new lot of the *p*-aminobenzyl glycoside of cellobiuronic acid was synthesized and the experiments repeated. The second preparation of antigen was administered as in the previous experiment to a group of three normal rabbits. After three courses of immunization one animal in this group failed to

show cellobiuronic acid antibodies, a second gave a moderate antibody response, whereas the serum of the third animal showed the presence of precipitins in high titre and was used in the following protection tests.

This serum was tested in mice for the presence of protective antibodies against Pneumococcus Types II, III, and VIII and the results of these experiments are given in Table III. The experiments were controlled by including a group of mice which received virulent organisms together with the serum of the same rabbit before immunization with the cellobiuronic acid antigen was begun.

The results presented in Table III confirm the observations recorded in Table II and in addition show that the cellobiuronic acid antiserum affords protection against infection with Type II pneumococci as well as with Types III and VIII organisms. This result clearly demonstrates that the artificial antigen containing the azobenzyl glycoside of cellobiuronic acid stimulates in rabbits the formation of antibodies capable of conferring passive immunity on mice against infection with a number of different types of virulent pneumococci. The significance of this finding will be discussed later.

B. Cellobiose Antiserum.—It will be recalled that the chemical structure of the two saccharides, cellobiose and cellobiuronic acid, is identical save for the grouping occupying the 12th position in each. Antigens containing the azobenzyl glycosides of these two saccharides give rise in rabbits to antibodies which show some serological crossing, yet are quite specific as shown by inhibition tests (1 *c*). In the present investigation it has been found that the antiserum elicited by the cellobiuronic acid antigen agglutinates Type III pneumococci and causes a definite swelling of the capsule. The antiserum to the cellobiose antigen, on the other hand, exhibits neither of these phenomena.

In order, therefore, to ascertain whether the cellobiose antiserum will confer passive protection against pneumococcal infections, the most potent cellobiose antiserum was tested in mice with virulent cultures of Types II, III, and VIII pneumococci by the method described. In no instance was any protective action observed. The results of these experiments, which are given in Table IV, again demonstrate the wide variance in immunological function of the anti-

bodies elicited by an antigen containing the disaccharide as opposed to the immune bodies evoked by the aldobionic acid antigen.

Active Immunity: A. Rabbits Injected with Cellobiose Antigen.— To ascertain whether the rabbits injected with the cellobiose antigen had acquired active immunity, six animals were infected, 12 days after the last injection of antigen, by the intradermal inoculation of

TABLE III

Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice*

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-2}	D 72	S	D 24	D 24	—	—
10^{-3}	S	S	D 72	S	D 24	D 24
10^{-4}	S	S	S	S	D 40	D 72
10^{-5}	S	S	S	S	S	S
10^{-6}	—	—	—	—	S	S
Controls†						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 48		D 48		D 40	
Virulence controls (no serum)						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 40		D 48		S	

* The serum of a rabbit immunized with the second preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† Mice received 0.2 cc. of serum of rabbit before immunization with cellobiuronic acid antigen was begun.

0.2 cc. of a blood broth culture of a rabbit virulent strain of Type III Pneumococcus. The virulence of the culture was such that 0.001 cc. injected intradermally killed normal control rabbits within 48 hours. The animals which had previously received the cellobiose antigen promptly developed massive edematous and necrotic lesions following infection and succumbed within 48 to 60 hours.

B. Rabbits Injected with Cellobiuronic Acid Antigens.—Four of the rabbits which had received the cellobiuronic acid antigen were likewise tested for active immunity. The intradermal inoculation of virulent Type III organisms was made 12 days after the last injection of antigen. All four animals developed marked lesions and ran a febrile course. In each instance save one, however, the lesions were smaller and less edematous than in the normal controls or in the animals which had received the cellobiose antigen. Three of the infected rabbits recovered from the dermal infection. One rabbit died within 72 hours.

TABLE IV

*Protective Action of Anticellobiose Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-5}	D 28	D 45	D 45	D 45	D 45	D 45
10^{-6}	D 47	D 72	D 47	D 47	D 45	D 45
Virulence controls (no serum)						
10^{-6}		D 45		D 45		D 40
10^{-7}		D 45		D 45		D 45
10^{-8}		D 45		D 45		D 45

* The serum of a rabbit immunized with cellobiose antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

The results of these experiments indicate clearly that rabbits immunized with the cellobiuronic acid antigen acquire definite resistance to intradermal infections with a virulent strain of Type III Pneumococcus. Animals injected with the cellobiose antigen, on the other hand, show no resistance whatsoever.

DISCUSSION

From the results of our immuno-chemical studies on uronic acid antigens, the concept has gradually evolved that it might be possible to confer on experimental animals immunity to pneumococcus infec-

tion with an artificial antigen containing a simple saccharide as the immuno-specific group instead of the more complex bacterial polysaccharide itself. Earlier studies showed that artificial antigens containing the azobenzyl glycosides of glucuronic and galacturonic acids, though reactive in antipneumococcal sera, failed to stimulate in various species of experimental animals immunity to pneumococcal infections (1 b). The reason for this failure may be attributed to the fact that the hexose uronic acids do not approximate closely enough in structure the aldobionic acids which constitute the fundamental building stones of certain of the type specific polysaccharides of bacterial origin. The structural unit of the Type III pneumococcus polysaccharide is cellobiuronic acid (2 a). This aldobionic acid is unusually suited for testing the hypothesis set forth above, since much of the basic research for such a study already has been accomplished, and the acid itself is readily procured from the acid hydrolysis products of the bacterial polysaccharide. From the results of the present investigation it has been proven beyond question that the aldobionic acid, functioning as the immuno-specific group of an artificial antigen, evokes in rabbits antibodies which have many properties in common with those elicited by an antigen containing the more complex capsular polysaccharide.

In a communication presented some years ago from this laboratory (3), it was shown that an artificial antigen containing the azobenzyl ether of the Type III capsular polysaccharide evoked in rabbits antibodies which specifically agglutinated Type III pneumococci, precipitated the homologous specific polysaccharide, and protected mice against infection with Type III organisms. Not only does the cellobiuronic acid antiserum precipitate the Type III capsular polysaccharide, when combined with egg albumin (1 c), and agglutinate Type III organisms, but the sera of animals immunized with the cellobiuronic acid antigen likewise confer passive protection on mice against infection with virulent Type III pneumococci.

It should not be inferred, however, that the antibodies evoked by the polysaccharide antigen or by heat-killed Type III pneumococci are identical with those elicited by the cellobiuronic acid antigen. The results of the specific inhibition tests presented in the previous communication (1 c) clearly demonstrate that the polysaccharide and cellobiuronic acid antibodies are similar but not identical since they

fail to show a complete reciprocal relationship. Furthermore the results of the experimental studies presented in this communication have brought forth a new and important principle. Whereas the antigen containing the complex bacterial Type III pneumococcus carbohydrate gives rise to antibodies which are type specific, those elicited by the antigen containing the pattern unit, or aldobionic acid show a broader specificity for they confer passive protection on mice not only against infection with Type III pneumococci but against Types II and VIII organisms as well.

Although cellobiuronic acid antiserum causes agglutination and *Quellung* only with the Type III Pneumococcus it must be borne in mind that protection tests are far more subtle than are these gross qualitative phenomena and that protection can be demonstrated with amounts of antibody which cannot be detected with other techniques. Furthermore, it has been proven that cellobiuronic acid is a constituent of the Type VIII pneumococcus polysaccharide. For these reasons, therefore, it is not out of the question that the protection which cellobiuronic acid antiserum affords mice against infection with Type VIII pneumococci can be attributed to the identity in structure of a portion of the polysaccharide molecule. The striking results obtained with Type II Pneumococcus cannot be explained until a more comprehensive understanding of the uronic acid constituent of the capsular polysaccharide of this microorganism is had. The results of the foregoing experiments indicate the importance of ascertaining the exact constitution of the specific polysaccharides of encapsulated pathogens, for it is only through such knowledge that the enigma of their specificities will be fully explained.

SUMMARY

1. An artificial antigen containing the azobenzyl glycoside of cellobiuronic acid gives rise in rabbits to antibodies which: (*a*) give the Neufeld reaction and agglutinate Type III pneumococci, (*b*) confer passive protection on mice against infection with Types II, III, and VIII pneumococci.

2. Rabbits immunized with the artificial cellobiuronic acid antigen acquire active resistance to infection with virulent Type III pneumococci.

3. The antibodies evoked by an antigen containing the azobenzyl glycoside of cellobiose exhibit none of these phenomena.

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ARTIFICIAL MAINTENANCE MEDIA FOR CELL AND ORGAN CULTIVATION

I. The Cultivation of Fibroblasts in Artificial and Serumless Media[*]

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Plate 19

The importance of developing artificial media that can be used in the place of serum for maintaining the life of tissues and organs outside the body hardly needs to be emphasized. Many of the studies for which the organ culture technique[1] was developed, as well as others that can be carried on by the simpler methods of tissue culture, depend for their success on the creation of suitable artificial media. These media are needed to reduce the cost of experimentation, to make possible extensive cultivation of human organs and those of small animals from which serum in sufficient quantity cannot be obtained, and for all studies in which the production of serum and other protein substances is to be investigated. For the latter purpose, media that are serumless and free from protein will be required. But for other work media that contain serum as one constituent may be used.

Several artificial media designed to promote rapid growth of cells in tissue culture have already been described.[2,3] But these are not

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[*] Reported in brief in *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 291.

[1] Carrel, A., and Lindbergh, C. A., *Science*, 1935, **81**, 621. Lindbergh, C. A., *J. Exp. Med.*, 1935, **62**, 409. Carrel, A., *J. Exp. Med.*, 1937, **65**, 515. Carrel, A., and Lindbergh, C. A., The culture of organs, New York, Paul Hoeber, Inc., 1938.

[2] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, **44**, 503; 1928, **47**, 353, 371; **48**, 533. Baker, L. E., *J. Exp. Med.*, 1929, **49**, 163; *Science*, 1936, **83**, 605.

[3] Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer*, 1933, **18**, 28; 1935, **24**, 393.

suitable for studies in which the functioning of organs and tissues is to
be investigated. For such studies media that will maintain cells
without promoting growth are required. The pupose of this report is
to describe media devised for this purpose, and the results obtained
when they were used to sustain the life of a pure strain of fibroblasts
in vitro. Experiments in which they were used for organ cultiva-
tion will be described in another communication.[4]

Composition of the Media

In the course of this work many different media with varying com-
binations of constituents have been used. For the sake of brevity
only four will be described. Their composition will be given first,
and then a description of the way in which they can be prepared.

Medium I.—

Whole blood digest, as described further on, in amount to give 30 to 60 mg.
per cent nitrogen

Serum	2 or 3	per cent
Phenol red	5 mg.	" "
Tyrode's solution		

Medium II.—

Whole blood digest to give either 30 or 60 mg. per cent nitrogen

per 100 cc.

Cysteine hydrochloride	9.0	mg.
Insulin	0.1	unit
Thyroxine	0.001	mg.
Hemin	0.004	mg.
Vitamin A(containing some D)[5] dissolved in serum	100.0	units
Vitamin B$_1$	0.1	gamma
Vitamin B$_2$	3.4	gammas
Ascorbic acid	0.3	mg.
Glutathione	1.2	mg.
Glucose	200 to 300	mg.
Phenol red	5.0	mg.
Potassium iodide	0.13	mg.
Salts as in Tyrode's solution		

[4] Some experiments in which organs have been cultivated in these media have
already been described by Carrel, A., and Lindbergh, C. A., The culture of organs,
New York, Paul Hoeber, Inc., 1938.

[5] The vitamin A was prepared from haliver oil and contained 1 unit vitamin
D for each 5 units of vitamin A.

Many of the constituents used in this medium were selected because they had previously been found either by Vogelaar and Erlichman[3] or by Baker[2] to prolong the life of cells in artificial, growth-promoting media. The concentrations of the individual constituents have been adjusted to those that seemed best suited to maintenance.

To bring the vitamin A into a form in which it could be taken up by the cells, it was dissolved at high concentration in serum. Then a small amount of this serum, about 0.07 per cent, was used in the medium.

Medium III.[6]—This contained all the constituents listed under medium II and in addition:

	per 100 cc.	
Tryptophane	5 to 10	mg.
Witte's peptone to give	6.0	mg. nitrogen
Sodium glycerophosphate	57.5	mg.
Urea	2.4	mg.
Glycerine	0.2	cc.
Thymus nucleic acid[7]	20.0	mg.
Antuitrin	0.2	cc.
Adrenalin chloride[8] (1 : 1000)	0.1	cc.
Eschatin (suprarenal[8] cortex hormone)	0.1	cc.
Pitressin[8] (pituitary hormone)	0.1	cc.

Medium IV.—This contained all the constituents used in medium III with the exception of the vitamin A. It was, therefore, a serumless medium.

Preparation of the Blood Digest.—Approximately 700 cc. of cow blood is obtained

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[6] Though many experiments have been made in the course of this work, with the individual constituents, to ascertain the nature of the substances needed and the concentrations at which each should be used, it was not always possible to arrive at final conclusions. Thus, nucleic acid was incorporated in medium III on some evidence obtained with it in simpler media but, after the completion of the experiments described here, some additional experiments of rather short duration were made in which medium III was used with and without the nucleic acid. These experiments indicated that nucleic acid at the concentration used made the cells more granular than they were when it was omitted. Probably, therefore, with further experimentation, it will be possible to devise media that are simpler and still more satisfactory than those developed thus far.

[7] The authors are indebted to Dr. P. A. Levene of The Rockefeller Institute, who was kind enough to prepare and furnish this substance.

[8] Excellent results have been obtained with the whole thyroid gland in a medium containing only 1/5 of this quantity of hormone.

from the slaughter house.   Coagulation is prevented during delivery by having
the blood collected in a bottle containing 50 mg. of heparin[9] dissolved in 20 cc.
of Ringer's solution.   450 cc. of this blood is shaken with 225 cc.[10] of chloroform
and placed in an incubator at 37°C. overnight.   The next day the solidified mass
is broken up, either by macerating it in a mortar, or passing it through a meat
chopper.   It is then diluted with distilled water to a volume of 6 liters.   N/1
sodium hydroxide solution is added in quantity sufficient to bring the pH of the
mixture as measured by the glass electrode to 10.2.[11]   Then 10 gm. of Armour's
pancreatin is added and the mixture is incubated for 24 hours, being shaken at
half hour intervals during the first few hours.   The next day, alkali is again
added to bring the pH to 8.3.   Then 4 gm. more pancreatin is added and digestion
is continued without further adjustment of pH for 2 days.   After this, the mix-
ture is filtered through glass wool, and then through filter paper.   The volume
of the filtrate is measured, and trichloracetic acid is added in quantity sufficient
to make its concentration 2.5 per cent.   After standing at room temperature for
16 hours, the clear, supernatant fluid is siphoned off, and the remaining cloudy
fluid centrifuged.   The siphoned fluid and that obtained on centrifuging are
combined and boiled in an open basin to approximately half their original volume.
This destroys the enzyme, decomposes the trichloracetic acid, and removes the
chloroform.   The cryoscopic point is determined and the fluid made isotonic by
addition of salt or water as required.   Total and amino nitrogen determinations
are made and the fluid is sterilized by autoclaving.   The ratio of amino to total
nitrogen of digests so prepared varies from 0.42 to 0.45.   Samples of the digest
saturated with ammonium sulfate show only the faintest trace of precipitate.

Preparation of Medium I.—To prepare 250 cc. of medium, calculate the amount
of blood digest required to furnish 75 or 150 mg. nitrogen (*i.e.*, 12.5 or 25 cc.
of a digest containing 600 mg. per cent nitrogen).   Add to this 5 or 7.5 cc. homol-
ogous serum and 1.25 cc. of a 1 per cent solution of the sodium salt of phenol red.
Then dilute with Tyrode's solution to 250 cc.

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[9] Obtained from the Connaught Laboratories, Toronto.

[10] 225 cc. of chloroform were used in making the digest for this work.   A sub-
sequent experiment has shown that by reducing this quantity to 50 cc., a digest
of the same characteristics is obtained.   Thus, when a given lot of blood was
divided into two parts, one of which was treated with 225 cc. of chloroform, and
the other with 50 cc., the following analytical results were obtained: Total nitro-
gen in the first digest, 439 mg. per cent; and in the second, 451 mg. per cent.
Ratio of amino to total nitrogen, 0.445 in the first, and 0.477 in the second.   As
yet no comparative tests of these digests for their ability to support cell life have
been made.

[11] In case a glass electrode is not available, it is advisable to add the pancreatin
before adjusting the pH, and then to add N/1 NaOH solution until the mixture is
just alkaline to phenolphthalein paper.

Preparation of Media II, III, and IV.—Stock solutions containing the most stable and inexpensive constituents at concentrations four times as great as those desired in the final medium are prepared first. These stock solutions are sterilized by passing them through a Berkefeld filter. They may be preserved at ice box temperature for 4 or 5 weeks. Solutions of the less stable and more expensive constituents are prepared in small quantity at frequent intervals. These are combined from time to time with small amounts of the stock solution to make the complete media. For organ cultivation, it is advisable to prepare a liter or more of the stock solution at a time. For culture work, much smaller quantities are advised. The freezing point of the medium should be determined before it is used and should lie between −0.62 and −0.66°C. The pH should be adjusted between 7.4 and 7.6. All media when completed should be sterilized by Berkefeld filtration.

To Prepare 1 Liter of Stock Solution for Medium II.—Calculate the volume of blood digest required to give 1200 or 2400 mg. nitrogen as desired (*i.e.*, 400 cc. of digest containing 600 mg. per cent nitrogen if a final medium containing 60 mg. per cent nitrogen is to be used). Measure out this volume and add:

Solution of sodium salt of phenol red, 1 per cent	20.0 cc.
Insulin, Squibb's (10 units per cc.)	0.4 cc.
Thyroxine-hemin solution containing 1 mg. thyroxine and 4 mg. hemin in 100 cc.	4.0 cc.
Potassium iodide, 2.8 per cent, diluted with water 1:100	18.6 cc.
Double strength Tyrode's solution	22.2 cc.
Cysteine hydrochloride, solid	360.0 mg.
Isotonic sodium bicarbonate solution, 1.4 per cent	6.8 cc.

Then dilute to 1 liter with Tyrode's solution modified to contain 300 mg. per cent glucose.

To Prepare 250 Cc. of Medium II.—Take 62.5 cc. of the stock solution just described and add:

	cc.
Ascorbic acid-glutathione solution (see below)	1.5
Vitamin B₁ (0.1 mg. per cent solution betaxin)	0.25
Vitamin B₂ (1.0 mg. per cent solution riboflavin)	0.85
Vitamin A serum, containing 1400 international units per cc.	0.18
(or that amount calculated to furnish 250 international units)	

Then dilute to 250 cc. with Tyrode's solution containing 300 mg. per cent glucose.

To Prepare 1 Liter of Stock Solution for Medium III.—Calculate the volume of blood digest needed to supply 2400 mg. nitrogen (or 1200 mg. nitrogen, if a final medium containing 30 mg. per cent nitrogen is desired). Add to this:

Tryptophane	200 or 400 mg.
Solution of sodium salt of phenol red, 1 per cent	20.0 cc.
Insulin, Squibb's (10 units per cc.)	0.4 cc.
Thyroxine-hemin solution containing 1 mg. thyroxine and 4 mg. hemin in 100 cc.	4.0 cc.
Cysteine hydrochloride, solid	360.0 mg.

Potassium iodide, 2.8 per cent solution diluted 1:100 with water	18.6 cc.
Urea	96.0 mg.
Vitamin B_1 (0.1 mg. per cent solution betaxin)	4.0 cc.
Vitamin B_2 (1.0 mg. per cent solution riboflavin)	13.6 cc.
Witte's peptone, 7.5 per cent solution in water	24.8 cc.
Sodium glycerophosphate	2.3 gm.
Glycerine, Kahlbaum's sp. gr. 1.23	8.0 cc.
Glucose	3.0 gm.
Sodium bicarbonate	270.0 mg.
Water, triple distilled	243.0 cc.

Then dilute to 1 liter with a Tyrode's solution from which the glucose has been omitted, and which contains sodium chloride at a concentration of 7.78 gm. per liter.

To Prepare 250 Cc. of Medium III.—Take 62.5 cc. of the stock solution just described and add:

Ascorbic acid-glutathione solution	1.5 cc.
Vitamin A serum containing 1400 international units per cc. (or that amount calculated to furnish 250 international units)	0.18 cc.
Antuitrin, Parke-Davis	0.5 cc.
Adrenalin chloride,[8] 1:1000 solution	0.25 cc.
Pitressin,[8] pituitary hormone, Parke-Davis	0.25 cc.
Eschatin,[8] suprarenal cortex hormone, Parke-Davis	0.25 cc.
Thymus nucleic acid dissolved in Ringer's solution with the aid of a few drops of N/1 NaOH	50.0 mg.
Water[12]	7.0 cc.

Then dilute to 250 cc. with Tyrode's solution containing 300 mg. per cent glucose.[10]

Phenol Red 1 Per Cent Solution of the Sodium Salt.—Weigh 1.000 gm. of phenol red. Grind this in a mortar with 28.2 cc. of exactly N/10 NaOH solution until it is all dissolved. Dilute to exactly 100 cc. with water, using a part of the water to transfer the dye to a graduated flask.

Thyroxine-Hemin Solution (Prepared as Described by Vogelaar and Erlichman).[3] —To 5 mg. thyroxine, add 6 cc. absolute alcohol, 2 cc. of 1 per cent NaOH, and 2 cc. water. Boil down to 3 cc. Add water to 10 cc. Then add 20 mg. hemin. Dilute 1:50 with water to obtain a solution having 1 mg. thyroxine and 4 mg. hemin in 100 cc.

Double Strength Tyrode's Solution.—A solution containing all the constituents of Tyrode's solution at twice the usual concentration. Sterilize by filtering.

Tyrode's Solution Modified to Contain 300 Mg. Per Cent Glucose.—Follow

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[12] The stock solution for medium III is hypertonic. Therefore water is added in making the medium. The stock solution cannot be made isotonic unless the volume of digest required does not exceed 554 cc.

directions for making ordinary Tyrode's solution, reducing the sodium chloride to 7.78 gm. per liter and increasing the glucose to 3 gm. Sterilize by filtering. It is sometimes convenient to make this solution without adding the glucose since such a solution can be preserved for a few days in the ice box without filtering. Then the glucose may be added as each stock solution or medium is made.

Ascorbic Acid-Glutathione Solution.—Dissolve 40 mg. glutathione (Hoffman-La Roche) and 10 mg. crystalline vitamin C (natural, Abbott Laboratories) in 20 cc. Ringer's solution. Sterilize by passing through a 1 inch Berkefeld filter. The presence of glutathione is necessary to stabilize the vitamin C and protect it from oxidation. The solution should be made fresh every 10 days or 2 weeks and preserved in the ice box.

Witte's Peptone Solution, 7.5 Per Cent.—15 gm. of Witte's peptone is added to 200 cc. triple distilled water in a pressure bottle. Then this is autoclaved for 15 minutes at 30 pounds pressure or 15 pounds gauge pressure.

Vitamin A Serum.—To prepare vitamin A serum, a potent concentrate of vitamin A must be obtained. This may be prepared as described by Baker[13] or it may be obtained commercially.[14] As soon as this concentrate is received, it should be divided into small lots of approximately 0.75 cc. each, and sealed in small tubes under an atmosphere of CO_2.

Before the vitamin is incorporated in the medium, it must be dissolved in serum. To do this, place approximately 0.5 cc. of the concentrate in a small Erlenmeyer flask and add 30 cc. of serum. Shake the flask violently for 20 or 30 minutes in a shaking machine so as to obtain a finely divided suspension of the vitamin concentrate. Then allow the mixture to stand overnight at room temperature. The next day, filter the serum through a Seitz clarifying filter, and then sterilize it by passing it through a 1 inch Berkefeld filter. Sera containing as much as 1800 to 2600 international units of vitamin A per cc. may be obtained in this way. The amount dissolved varies with the individual sera and the species of animal from which it is taken.

To protect the vitamin A serum from oxidation it is divided into portions 1 to 2 cc. in volume, distributed in small glass tubes, and sealed under CO_2. If all the oxygen is removed, the vitamin serum will retain its original potency for 3 or 4 months. When the serum is kept in stoppered tubes without removing the oxygen, approximately half the vitamin is lost in a week. Moreover, the resulting oxidation product is somewhat toxic.

The concentration of vitamin A in the serum may be determined by a method worked out in this laboratory by La Rosa. The procedure depends on the development of the typical blue color of the Price-Carr reaction. This is changed to a purplish red color on heating. The latter color is compared with a series of

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[13] Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1935, 33, 124.

[14] That used throughout this work was prepared and donated for this purpose by the Abbott Laboratories.

standards made up of sodium alizarin sulfonate. Details of the method are described by Parker.[15]

General Procedure Used in Testing the Media

The procedure used in testing these media for their ability to maintain fibroblasts was as follows: Cultures from a 26 year old strain of chicken heart fibroblasts were embedded in Carrel D-3 flasks in coagula containing 0.25 cc. chicken plasma and 0.75 cc. of the medium being used. To remove the serum from this coagulum, the cultures were washed on the following day and every 2 days thereafter for 2 hours at 37°C. with 2 cc. of the medium. Then this wash fluid was withdrawn and 0.5 cc. of new medium was supplied. The serum originally present in the coagulum disappeared under this treatment within 12 to 14 days.[16] The washing was continued, nevertheless, throughout the entire period of cultivation. Before the flasks were sealed, the pH of the medium was brought to 7.4 by using a gas mixture containing 3 per cent CO_2, 21 per cent O_2, and 76 per cent N_2. To ascertain the effect of the various media, the cells were examined microscopically at frequent intervals. Then, at the end of the cultivation period, which extended from 43 to 56 days, the vitality of the cells and their ability to proliferate were tested by transplanting them into a growth-promoting medium (plasma and embryo juice). A sister colony was cultivated in each case in a control medium the nature of which is indicated under each experiment.

RESULTS

Medium I.—When tested in the manner just described, medium I was found to be an excellent maintenance medium. Fibroblasts culti-

[15] Parker, R. C., The methods of tissue culture, New York, Paul B. Hoeber, Inc., 1938.

[16] To determine the length of time required to remove all this serum, a large number of coagula were prepared in Carrel flasks using several different samples of plasma diluted with Tyrode's solution to 25 per cent concentration. Then these were washed with Tyrode's solution in exactly the same manner as the cultures were washed with the medium. Each washing was followed by an incubation period during which 0.5 cc. of Tyrode's solution remained on the coagulum. After the third, fourth, fifth, sixth, and seventh washings some of the coagula were ground and the expressed fluid was analyzed for nitrogen. The organic matter was destroyed by digesting with sulfuric acid and hydrogen peroxide in the presence of selenium oxychloride, and the ammonia was determined by nesslerization. It was found that approximately two-thirds of the serum was removed by each washing and incubation period. After five washings, nitrogen equivalent to 0.001 cc. of serum was found. After seven washings, the largest amount of serum found was 0.0002 cc. Therefore, it is probably safe to assume that the serum is reduced to a negligible quantity by the end of 12 days' cultivation, and is completely removed soon after the end of the 2nd week.

vated in it remained alive and in good condition for 43 days.[17] During the first few days, *i.e.*, while considerable serum was still present in the coagulum, the cells proliferated at a very slow rate. After the concentration of the serum was reduced to that of the nutrient fluid, they were maintained with little or no proliferation. Then, when a little plasma was added on the 43rd day, to reinforce the coagula, the cells began to proliferate again. Control colonies that were cultivated in blood digest and Tyrode's solution died soon after all the serum had been removed from the coagulum. The colonies kept in digest supplemented with serum at 1 per cent concentration lived
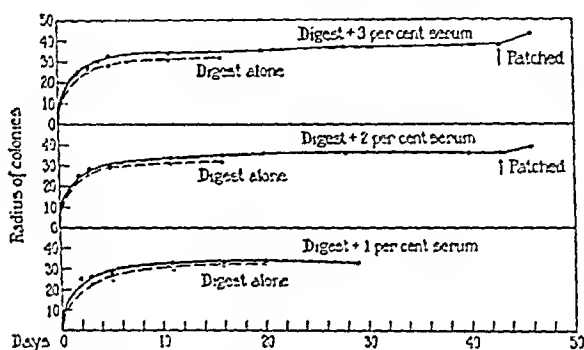
TEXT-FIG. 1. Experiment 10816-C. Comparison of the rate of growth and the duration of life of fibroblasts cultivated in blood digest, supplemented with 1, 2, and 3 per cent serum, with that of sister colonies cultivated in blood digest without serum. Nitrogen concentration of the blood digest, 60 mg. per cent. The increase in growth on the 43rd day is due to patching the coagula with a small amount of plasma. Radius in mm. × 16.

longer than those kept in digest alone, but not as long as those that received serum at 2 or 3 per cent concentration with the digest. Photographs illustrating the condition of the cells that were cultivated in blood digest alone, in blood digest supplemented with 1 per cent serum, and in blood digest supplemented with 2 and 3 per

[17] In another experiment in which blood digest and Tyrode's solution were the only substances supplied in the nutrient fluid but in which the coagulum was reinforced once in 2 or 3 weeks by adding 2 drops of plasma, the tissue remained alive and in good condition for 70 days and proliferated again on being transferred to a growth-promoting medium.

cent serum (medium I) are shown in Figs. 1 *a* to 1 *d*.   Growth curves showing the duration of life of these cultures and that of the control colonies are shown in Text-fig. 1.

Medium II.—Chicken heart fibroblasts cultivated in medium II lived for 50 days. The control colonies, cultivated in blood digest diluted with Tyrode's solution to the same nitrogen concentration as that used in the medium, died during the 3rd week of cultivation. The cells in the experimental medium remained in good condition for 6 weeks. During the 7th week of cultivation, the cells at the periphery of the colony became somewhat scattered and began to look starved. As it seemed probable that longer cultivation in this medium would not be feasible, the colonies were transferred on the 50th day to a new coagulum and given growth-promoting nutrients. Active proliferation ensued. It would seem, therefore, that this medium can maintain the cells for a considerable time, but not indefinitely. Photographs illustrating the condition of the cells in medium II when made with digest at a nitrogen concentration of 30 mg. per cent, and also when containing digest at a nitrogen concentration of 60 mg. per cent, are shown in Figs. 2 *a* and 2 *b*. Growth curves showing the duration of life of colonies cultivated in these media and that of sister cultures kept in digest and Tyrode's solution are shown in Text-fig. 2. The second curve in each case is that of the experimental colony after it was transplanted and given a growth-promoting medium.

Medium III.—This was devised in an attempt to improve medium II. Four of the ten new constituents that were added, antuitrin, tryptophane, Witte's peptone, and sodium glycerophosphate, were found, when added separately to medium II, to improve the nutritive and maintenance value of that medium. Beneficial action was also observed with the other six substances when they were used together. But the differences observed when each was tested separately were too small to constitute definite proof that they were all essential. To illustrate the effect of the complete medium, an experiment is cited in which a comparison was made of the maintenance of power of medium III and that of medium II already improved by the addition of antuitrin and tryptophane. Sister colonies of fibroblasts were cultivated in these two media for 49 days. Almost from the begin-
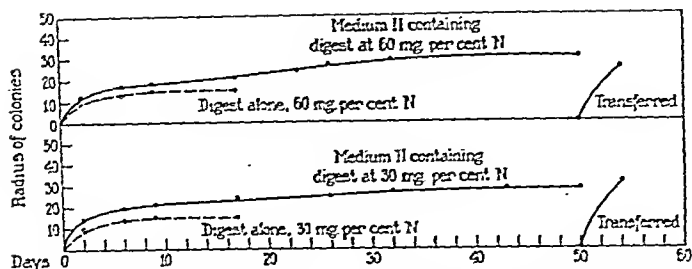
TEXT-FIG. 2. Experiment 10835-C. Comparison of the rate of growth and duration of life of fibroblasts, cultivated in artificial medium II, with that of sister colonies cultivated in blood digest and Tyrode's solution, showing growth of the former after transplantation on the 50th day into a growth-promoting medium. Nitrogen concentration of the blood digest, 30 and 60 mg. per cent.
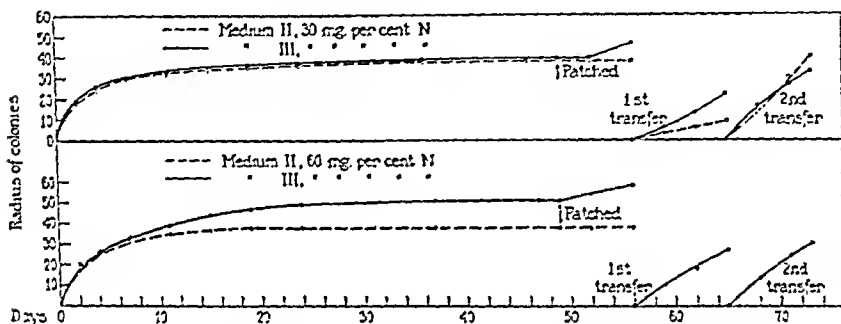
TEXT-FIG. 3. Experiment 10915-C. Comparison of the rate of growth and the duration of life of fibroblasts cultivated in artificial medium II to which antuitrin and tryptophane have been added, with that of sister colonies cultivated in artificial medium III showing growth in all cases in medium III and in some cases in medium II after transplantation on the 56th day into a growth-promoting medium. The increase in the rate of growth on the 49th day was due to patching the coagulum with a small amount of plasma. Nitrogen concentrations, 30 and 60 mg. per cent.
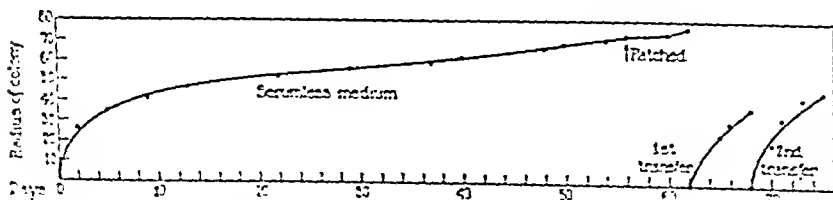
TEXT-FIG. 4. Experiment 11004-C. Curve showing the rate of growth and duration of life of a colony of fibroblasts cultivated for 56 days in an artificial and serumless medium (medium IV), showing growth after transplantation on the 62nd day to a growth-promoting medium. Nitrogen concentration, 60 mg. per cent.

ning of the experiment, the colonies in the more complete medium presented a better appearance. The tissue was thicker and the cells larger and clearer than were those cultivated in the simpler medium. In the experiments in which the digest was used at a nitrogen concentration of 60 mg. per cent, an exceedingly slow growth was observed. After 7 weeks of cultivation, the cells in the simpler medium began to look starved, as they had in the experiments with medium II cited above, while those in the more complete medium still seemed to be well nourished. On the 49th day, a small amount of plasma was added to reinforce the coagula. The colonies that had been given the more complete medium responded to this treatment by increasing in size. Those given the simpler medium did not respond. On the 56th day, the colonies were transferred to new coagula and given growth-promoting nutrients. All of those that had been cultivated in medium III grew actively, while only 25 per cent of those that had received the simpler medium were able to proliferate. Growth curves showing these results are reproduced in Text-fig. 3. Photographs illustrating the condition of the cells in medium III are shown in Fig. 3.

Medium IV.—This medium is serumless. It differs from medium III only by the omission of vitamin A and that small amount of serum that was required to dissolve it. Vitamin A had been incorporated in media II and III because it is a normal constituent of serum and because it had also been found to be an essential constituent of artificial, growth-promoting media. But no evidence indicating that it is essential to maintenance has been obtained. To ascertain, therefore, if it, and the serum that had been used to dissolve it, might be eliminated from the maintenance medium, an experiment was made in which sister colonies of fibroblasts were cultivated in medium III made up with and without vitamin A. The colonies that were kept in the serumless medium lived for 56 days. And the cells within those colonies remained a little cleared and appeared to be in better condition than those that had the vitamin and serum at their disposal. On the 56th day of cultivation, a little plasma was added to reinforce the coagula. Then on the 62nd day the cells were transplanted and given a growth-promoting medium. Those that had been maintained in the serumless medium as well as

those that were given the medium containing the vitamin responded by proliferating actively. A curve showing the duration of life and the slow growth of one of the colonies in the serumless medium is reproduced in Text-fig. 4. The second and third curves show the growth of this colony on two successive transfers. A photograph illustrating the condition of the cells cultivated in the serumless medium is given in Fig. 4. As will be seen, the cells in this medium have become quite large and show less polarity than those in the other media. However, when they were transplanted and given growth-promoting nutrients, they reverted immediately to their original form.

SUMMARY

Several media designed for maintaining the life of cells and organs outside the body have been described. Cultures from a pure strain of fibroblasts have been maintained in these media in vital condition and with little or no proliferation for periods varying from 43 to 56 days.[18] One of these media is very simple, inexpensive, and easy to prepare; and one is serumless.

[18] 12 to 14 days should be deducted to calculate the time the tissues lived in the absence of serum.

EXPLANATION OF PLATE 19

Fig. 1 a. Control culture. Fibroblasts cultivated 32 days in blood digest and Tyrode's solution, showing degenerated cells.   × 206.

1 b. Fibroblasts cultivated 32 days in blood digest, Tyrode's solution, and 1 per cent serum, showing cells in the process of degeneration.   × 206.

1 c. Fibroblasts cultivated 38 days in blood digest, Tyrode's solution, and 2 per cent serum (medium I).   × 206.

1 d. Fibroblasts cultivated 38 days in blood digest, Tyrode's solution, and 3 per cent serum (medium I).   × 206.

Nitrogen concentration of the blood digest, 60 mg. per cent in each case.

Fig. 2 a. Fibroblasts cultivated 37 days in medium II containing the blood digest at a nitrogen concentration of 30 mg. per cent.   × 206.

2 b. Fibroblasts cultivated 32 days in medium II containing the digest at a nitrogen concentration of 60 mg. per cent.   × 206.

Fig. 3. Fibroblasts cultivated 32 days in medium III containing blood digest at a nitrogen concentration of 60 mg. per cent.   × 206.

Fig. 4. Fibroblasts cultivated 56 days in a serumless medium (medium IV), containing the blood digest at a nitrogen concentration of 60 mg. per cent.   × 206.
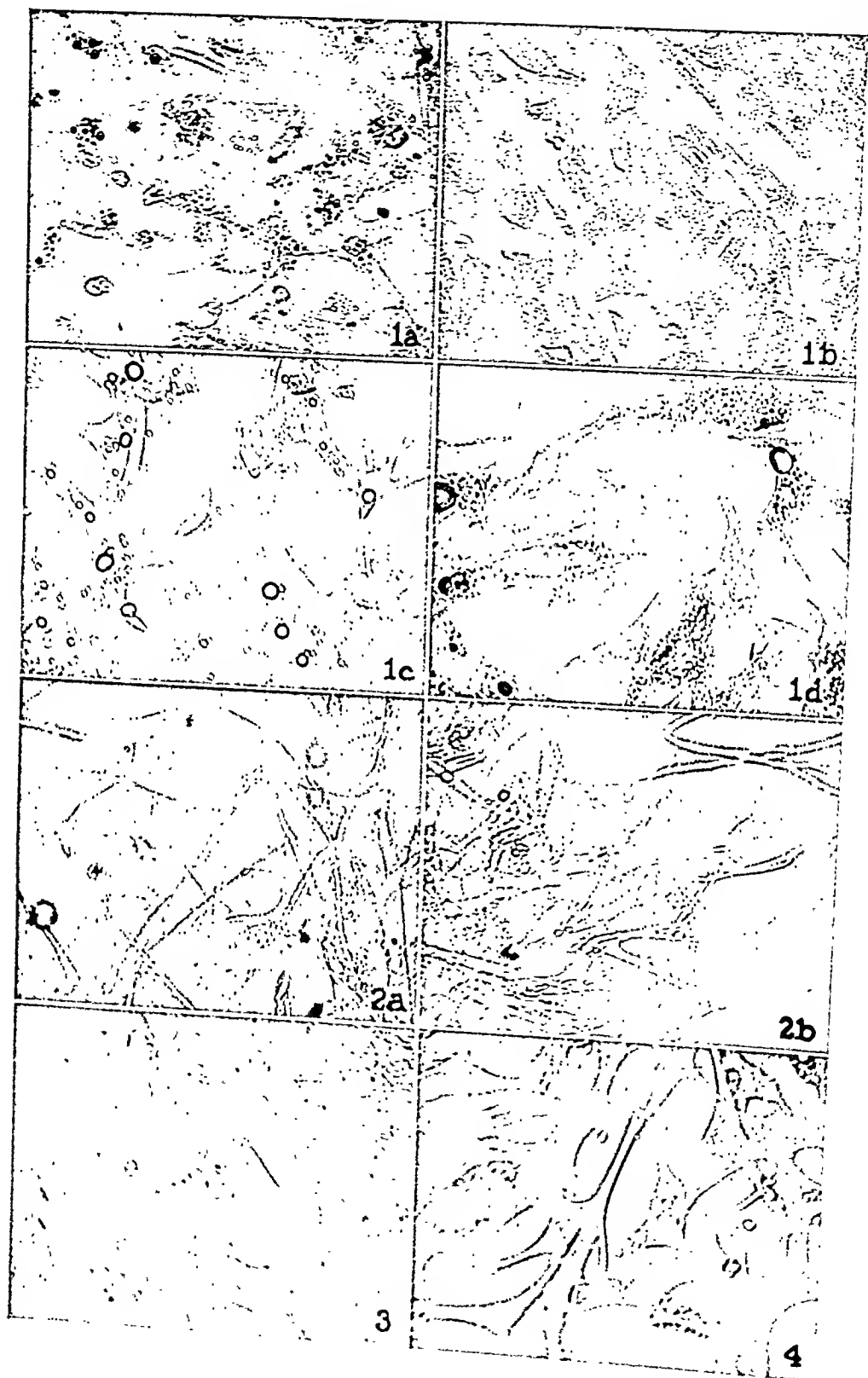
PLATE 19

COMPLEMENT FIXATION IN HUMAN MALARIA WITH AN ANTIGEN PREPARED FROM THE MONKEY PARASITE PLASMODIUM KNOWLESI

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The development of specific complement-fixing antibodies in the sera of monkeys infected with the malarial parasite *Plasmodium knowlesi* has been reported in a previous paper (1). Attempts to obtain a similar specific complement fixation reaction with sera from human beings with *Plasmodium vivax* and *Plasmodium falciparum* infections have, in the past, led to inconclusive results because no sensitive, specific, and easily standardized antigen was available. The intense infection produced in *rhesus* monkeys by *P. knowlesi* provides an abundant source of parasites for the preparation of an antigen which gives a definite and specific reaction with malarial sera from monkeys. This paper will describe the use of this *P. knowlesi* antigen in complement fixation tests with sera from human beings infected with *P. knowlesi* and also with the sera of those infected with *P. vivax* and *P. falciparum*. Because most of the human sera tested came from patients being treated for paresis by malarial therapy, it was necessary to eliminate certain non-specific reactions. This necessitated the preparation of antigens of the greatest possible specificity and the performance of many control tests with luetic and normal sera from persons with no malaria, and tests with antigens prepared from normal monkey cells. After the selection of a suitable antigen the intensity and duration of the complement fixation reaction due to malaria was studied.

The literature concerning various serological reactions in malaria and other protozoal infections of man and animals has been reviewed in previous papers (1, 2). In relation to the present studies the work of Kingsbury (3) on comple-

ment fixation in human malaria is of particular interest. This author used antigens prepared from the blood and internal organs of human beings with heavy *P. vivax* and *P. falciparum* infections. The most sensitive and specific antigen was obtained by laking washed parasitized blood corpuscles. With the antigens prepared from *P. falciparum* 48 per cent of the *falciparum* sera and 50 per cent of the *vivax* sera gave positive reactions. With *P. vivax* antigen 31 per cent of the *falciparum* sera and 67 per cent of the *vivax* sera gave positive reactions. These results indicate that the complement-fixing antibody is not species-specific. Kingsbury reported non-specific reactions due to differences in blood group of the antigen and the serum, and a considerable proportion of doubtful or positive reactions between luetic sera and all the antigens, especially those made from spleen, cerebral cortex, and liver. The question of serological reactions between malarial antigens and luetic sera has been reinvestigated during the course of the present work. There are many reports of positive reactions between Wassermann or Kahn antigens and non-luetic malarial sera. As many as 50 per cent of sera tested at the height of the acute malarial infection have given positive Wassermann or Kahn reactions when these same sera tested before the inoculation with malaria were negative (4). Other investigators have denied that malaria *per se* will cause a positive Wassermann (8, 9).

Materials and Methods

Antigens.—Tests with four different malarial antigens will be described in this paper. Two of these were prepared from the blood and two from the spleens of monkeys dying of acute infection with *P. knowlesi*. The parasitized blood cells were concentrated, frozen, dried, and preserved in sealed tubes according to the methods described in a previous paper (1). The spleens were dried in a similar manner. Details of preparing the antigen solutions follow.

Antigen 1 was prepared from dried parasitized blood cells by rehydrating the dried equivalent of 1 cc. of packed cells with 10 cc. of saline. The resulting suspension was then frozen and thawed four times, centrifuged, and the supernatant used as antigen.

Antigen 2 was prepared as previously described (1) from dried parasitized cells by grinding in a ball mill and extracting with saline. The proportion of dried cells to saline was the same as for antigen 1.

Antigen 3 was prepared from dried spleen in the same way as antigen 2, using 1 gm. of dried spleen to each 10 cc. of saline.

Antigen 4 was prepared from dried spleen by rehydrating with saline in the proportion of 10 cc. to each gram of dried material and freezing and thawing as with antigen 1.

In addition to these malarial antigens, an antigen was prepared from normal monkey red cells in the same way as antigen 1. This antigen was used for a normal control in the tests with malarial sera. It is designated as antigen N.

Antigen 1 was not anticomplementary undiluted and was used in the tests at a

dilution of 1:4. Antigen 2 was slightly anticomplementary undiluted and was used at a dilution of 1:10. Antigen 3 was definitely anticomplementary undiluted and was used at a dilution of 1:10. Antigen 4 was anticomplementary at a dilution of 1:2 and was used at a dilution of 1:12. Antigen N was not anticomplementary undiluted. None of these antigens showed any hemolytic properties when tested with sensitized sheep cells.

Sera.—Practically all of the tests described in this paper were made with sera from patients with paresis who were being treated by induced malarial infections with *P. knowlesi*, *P. vivax*, or *P. falciparum*. Part of the sera were collected by us at the Manhattan State Hospital, and part were furnished by Dr. Mark F. Boyd of the Station for Malaria Research at Tallahassee, Florida. Nine non-luetic malarial sera were also sent by Dr. Boyd. Specimens were taken during the clinical attack and at various times up to 6 or more months afterward. From twelve patients series of samples were taken at intervals before, during, and after the malarial attack. Many of the patients, especially those with *vivax* and *falciparum* malaria, were treated with quinine after the first course of malarial paroxysms. Luetic sera from patients presumably free of malaria were collected at various clinics in New York City.

Method of Performing the Complement Fixation Test.—The tests with human sera were made by the same procedure as that already described in detail for monkey sera (1) except that 2 units of complement were used instead of $2\frac{1}{2}$ units as for the monkey sera. This makes the test with human sera somewhat more sensitive. Except where it was desired to know the titer, the sera were not run at various dilutions but only in amounts of 0.1 cc., and the results were recorded as $\pm$, $+$, $++$, $+++$, and $++++$, according to amount of unhemolyzed cells remaining.

Standardization of Antigens by Serological Measurements of the Relative Amounts of Specific and Non-Specific Material.—In complement fixation tests with malarial sera requisites of a suitable antigen are: (a) low anticomplementary activity, (b) relatively low content of non-specific material, and (c) relatively high content of specific malarial antigen. For the purpose of measuring the relative amounts of specific (malarial) and non-specific (monkey) antigens in preparations of parasitized blood and parasitized spleen, a method of serological titration was devised. The specific antigen was titrated against hyperimmune monkey serum. In this reaction non-specific factors may be considered negligible. The amount of reactive monkey protein was measured by titration against the serum of a rabbit immunized with normal monkey erythrocytes. The method and results are illustrated in Table I where two antigens, one from blood and one from spleen, are compared.

With the anti-monkey (rabbit) serum, antigen 4 gives fixation of complement at a dilution which is at least four times the highest dilution of antigen 1 which gives a positive reaction. With the antimalarial (monkey) serum, antigen 4 is slightly less reactive than antigen 1.

This indicates that antigen 4 contains more non-specific material (monkey

protein) per reacting unit of malarial antigen than does antigen 1. Also, antigen 4 is more anticomplementary than antigen 1. Although it is possible that these reactions of complement fixation with monkey and rabbit sera are not entirely

TABLE I

Measurements of Relative Amounts of Specific (Malarial) and Non-Specific (Monkey) Antigens in Parasitized Blood, Parasitized Spleen, and Normal Blood

Serum		Antigen No.	Dilution of antigen							
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Anti-monkey (rabbit)	1:8	1 (blood)	++++	++++	++++	++++	+++	+	−	−
" "	1:8	4 (spleen)	++++	++++	++++	++++	++++	++++	++++	+++
" "	1:8	N (blood)	++++	++++	++++	++++	++++	+++	++	−
Antimalarial (monkey)	1:10	1 (blood)	++++	++++	++++	++++	++	−	−	−
" "	1:10	4 (spleen)	++++	++++	++++	+++	±	−	−	−
" "	1:10	N (blood)	−	−	−	−	−	−	−	−
None (saline)		1 (blood)	−	−	−	−	−	−	−	−
" "		4 (spleen)	++++	±	−	−	−	−	−	−
" "		N (blood)	−	−	−	−	−	−	−	−

TABLE II

Tests for Complement Fixation by Malarial Antigens with Luetic and Normal Sera

Antigen No.	Sera		Total number of sera tested	Reactions, per cent of total			
	Source	Wassermann		++ to ++++	+	±	−
1 (blood)	Lues	Positive	39	3	5	15	77
" "	"	Negative	13	8	8	8	76
" "	Normal	"	37	0	8	11	81
2 (blood)	Lues	Positive	31	0	13	10	77
" "	"	Negative	13	0	8	15	77
3 (spleen)	"	Positive	29	35	13	17	35
" "	"	Negative	13	16	8	16	60
" "	Normal	"	53	8	6	8	79
4 (spleen)	Lues	Positive	33	6	9	15	70
" "	"	Negative	13	8	16	8	68

comparable to reactions with human serum, this method appears to be useful as a preliminary test for selecting a suitable antigen, and it eliminates the time-consuming process of testing many antigens against many human sera. Furthermore, the results with this method are confirmed by the tests with human sera shown later in Table II and Fig. 1.

In Table I it may be seen that antigen N prepared from normal monkey erythrocytes reacts to a slightly higher titer with the anti-monkey serum than did antigen 1 which was prepared in the same way from parasitized cells. In the complement fixation tests with human sera both antigens were used at a dilution of 1:4. The sensitivity of antigen N to substances reactive with the monkey cells should, on the basis of the results with rabbit serum, be slightly greater than the sensitivity of antigen 1. In this way false positive reactions due to constituents of the red cells can be detected.

The standardization of malarial antigens and of normal control antigens can be accomplished with reasonable accuracy when the methods just described are used in conjunction with tests against malarial and non-malarial human sera. When a new preparation of antigen is made, this is compared with the old antigen by parallel tests, and the concentration is then adjusted so that the new antigen gives reactions of similar sensitivity and specificity.

Tests of Malarial Antigens for Non-Specific Complement Fixation with Luetic and Normal Human Sera

The four malarial antigens, prepared as described in the section on materials and methods, were tested with normal human sera and with Wassermann-positive and Wassermann-negative luetic sera. For the most part, each individual serum was tested with each of the four antigens. However, in some cases there was only enough serum to do tests with two or three of the antigens. The results of these complement fixation tests are summarized in Table II.

Antigens 1 and 2, prepared from parasitized blood cells, gave less frequent and weaker positive reactions than antigens 3 and 4, prepared from malarial spleens. Only two sera gave reactions stronger than + with antigen 1, and none of the sera tested gave reactions of + + or greater with antigen 2. With antigens 1 and 2 approximately the same percentage of + and ± reactions occurred with luetic as with normal sera. With antigen 3 a larger number of positive reactions was obtained with Wassermann-positive than with Wassermann-negative luetic sera, and the percentage of positive reactions with normal sera was considerably lower, being about the same as with antigens 1 and 2. These results indicate that antigen 3 contained considerable amounts of substances which fix complement in parallel with the Wassermann reaction. Antigen 4, also prepared from spleen, did not give a greater percentage of positive reactions with Wassermann-positive than with Wassermann-negative luetic

sera, but the proportion of positive reactions with these sera was slightly higher than was given by antigens 1 and 2 from blood.

It will be seen that antigen 3 cannot be considered reliable for tests with malarial sera which come from patients suffering from paresis, because of its reactivity with non-malarial luetic sera. The other three antigens gave less cross reaction with luetic sera, and with these antigens a reaction of ++ with serum in amounts of 0.1 cc. or less could be considered significant.

Serological Reactions of Normal Monkey Red Cells with Normal, Luetic, and Malarial Human Sera

Several investigators have recorded an agglutination of the red cells of *rhesus* monkeys by some human sera. This is apparently due to hetero-agglutinins not related to the human blood groups or to the Forssman antigen (5). In the present work, some of the patients with *P. knowlesi* infection had been inoculated with the infected blood of *rhesus* monkeys, and their sera showed definite agglutination of monkey cells. The presence of normal hetero-agglutinins in human sera apparently accounted for several other examples of strong agglutination occurring after 2 hours at 37°C. and numerous instances of weaker agglutination of monkey cells which occurred only after 24 hours in the ice box. It was, of course, necessary to determine to what extent these reactions affected the complement fixation with monkey antigens and human sera in the detection of malarial antibodies.

The results of complement fixation tests with the antigen prepared from normal monkey erythrocytes and with human sera from normal individuals and patients with syphilis and malaria are presented in Table III. The antigen was prepared and standardized for sensitivity by titration against anti-monkey rabbit serum as described in previous sections, and was used at a dilution of 1:4. Of the total of 150 sera tested, only one gave a reaction greater than + in an amount of 0.1 cc. with the normal monkey antigen. In the incidence of positive (+) and doubtful (±) reactions there was no important difference between luetic sera giving a positive Wassermann, those giving a negative Wassermann, and the normal sera. In the three series of malarial sera the percentages of positive and doubtful reactions were considerably higher than with the non-malarial sera. This suggests that malarial infection stimulates the production of hetero-antibodies which react with normal monkey cells, and in two or three cases the apparent development of such antibodies at low titer during the course of the malarial paroxysms was observed in patients who had not received monkey blood. These observations demonstrate the importance of using, in complement fixation tests for malaria, a control antigen made from normal cells in the same way and used at the same effective dilution as the malarial antigen.

On twenty-one non-malarial sera and twenty-eight malarial sera, agglutina-

tion and complement fixation tests with normal monkey cells were run in parallel. Of the total of forty-nine sera, twenty-eight (fourteen in each group) gave negative reactions in both tests. With five malarial sera and five non-malarial sera, the agglutination test was positive and the complement fixation test negative, and with nine malarial and one non-malarial sera both tests were positive. One serum gave weak complement fixation but no agglutination. These results indicate that with a fair proportion of sera the hetero-antibodies that cause agglutination of normal monkey cells react weakly or not at all in the complement fixation test.

<div align="center">TABLE III</div>

Tests for Complement Fixation by an Antigen Prepared from Normal Monkey Red Cells (Antigen N) with Normal, Luetic, and Malarial Human Sera

Source of sera	Total number of sera tested	Reactions, per cent of total			
		++ to ++++	+	±	−
Normal.....................................	37	0	6	6	88
Lues (Wassermann-positive)................	39	0	3	10	87
Lues (Wassermann-negative)...............	13	0	8	0	92
P. knowlesi infection and lues..............	25	0	12	24	64
P. vivax infection and lues.................	22	0	14	23	63
P. falciparum infection and lues............	14	7	7	29	57

Detection of Pseudopositive Reactions with "Normal" Antigen

According to the results which are presented in Table III, strong complement fixation reactions due to the antigens in normal monkey cells are rare. Consequently, strong reactions with a malarial antigen prepared from parasitized red blood cells may be considered significant. When the reaction with malarial antigen is weak or doubtful, the control with normal antigen is of value in eliminating a certain number of false positives.

In Table IV are presented results showing the relative intensity of false positive reactions using normal and luetic sera with normal monkey blood and the four previously described malarial antigens. Only those sera which gave a definitely positive reaction with more than one antigen are included in the table. In general, pseudopositive reactions were more frequent and stronger with the antigens prepared from spleen than with the antigens prepared from blood. Three sera, Gz, Ml, and Rg, were exceptional in giving positive reactions

with the blood antigens and negative reactions with the spleen antigens. Of the fourteen sera which gave positive or doubtful reactions with the malarial antigens prepared from parasitized red cells, six also gave reactions of similar intensity with the antigen N, prepared from normal red cells. Two sera, Gs and Gz, gave strong pseudopositive reactions with the antigens from malarial blood but none with

TABLE IV

Relative Intensity of False Positive Reactions with Various Malarial Antigens and Normal and Luetic Sera

Human serum	Antigen N (blood)	Malarial antigens				Wassermann
		1 (blood)	2 (blood)	3 (spleen)	4 (spleen)	
Bi	−	−	−	+	++	++++
Fl	−	+	±	++		+
Gs	−	++	−	++	++	++++
Ha	±	±	−	++	±	++++
Lk	−	±	−	++	−	++++
Lm	−	−	−	++	+	+
Lz	−	−	±	++++	++++	++++
Pc	−	−	−	++	±	++
Ss	±	±	+	+++		++++
Do	−	−	−	+	+	−
Gz	−	+++	+	−	−	−
Ml	+	+	−	−	−	−
Pr	±	±	±	++	+++	−
Bs	−	±		+++	+	Normal serum
Gr	−	−		+	++	" "
Kl	−	+		++	++	" "
Lr	+	+		+	+	" "
Rg	+	+		−	−	" "
Zl	−	±		++++	+	" "

the antigen from normal blood. Nine sera gave strong false positive reactions with one or both of the spleen antigens but no reaction at all with antigen N. From these results it is evident that the control tests run with antigen N are of value in eliminating about half of the false positive reactions when parasitized blood is used for the test, but such controls are of little value in eliminating the more frequent false positive reactions with spleen antigen.

Complement Fixation with P. knowlesi Antigens and Sera from Patients
Infected with P. knowlesi, P. vivax, and P. falciparum; Sensitivity
and Specificity of the Antigens

Complement fixation tests with sera from paretic patients receiving malaria therapy were done with four antigens prepared from the blood or the spleens of monkeys dying of *P. knowlesi* infection.   The results are summarized in Fig. 1.   Control tests with antigen N, prepared from normal monkey erythrocytes, were run in parallel with the tests of the malarial sera with antigen 1.   Several sera gave weak positive reactions of equal intensity with antigen 1 and antigen N.   These were recorded as negative.   When the reaction with antigen 1 was + or ++ and that with antigen N ±, the result was recorded as doubtful.   Stronger reactions with antigen 1 were recorded as ++ to ++++, depending upon whether the reaction with antigen N was negative, ±, or +.   Similar controls with antigen N were run with the luetic and normal sera from patients with no malaria, and in the corrected results shown in Fig. 1 about one-third of the pseudopositive reactions have been eliminated.[1]   Antigens 2, 3, and 4 were tested with the same sera as antigen 1, but no control tests with normal antigen were done.   The results with these antigens have, therefore, been recorded as positive or negative, regardless of what reaction the sera gave with antigen N.

From the results shown in Fig. 1, it is obvious that antigens prepared from *P. knowlesi* fix complement not only with homologous antisera but also with sera from *P. vivax* and *P. falciparum* infections. In fact, antigens 2 and 4 gave a higher percentage of fixation with *vivax* and *falciparum* sera than with *knowlesi* sera.   Antigens 1, 2, and 4 gave significantly higher percentages of positive reactions with the three kinds of malarial sera than with sera from normal individuals or from those with syphilis alone.   Antigen 3 gave a relatively high percentage of strongly positive reactions with *knowlesi* and *vivax* sera, but there were also many cross reactions with the luetic and normal sera.   Although antigens 2 and 4 gave relatively few positive reactions with non-malarial luetic sera, the positive reactions with

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[1] The results for antigen 1 recorded in Table II include all of the positive reactions with this antigen, irrespective of the reaction of these sera with antigen N.

malarial sera were generally weaker than those of antigens 1 and 3. With the malarial sera taken as a group, antigen 1 was superior both in specificity and sensitivity to the other three antigens, and with the homologous anti-*knowlesi* sera this antigen gave the highest per-
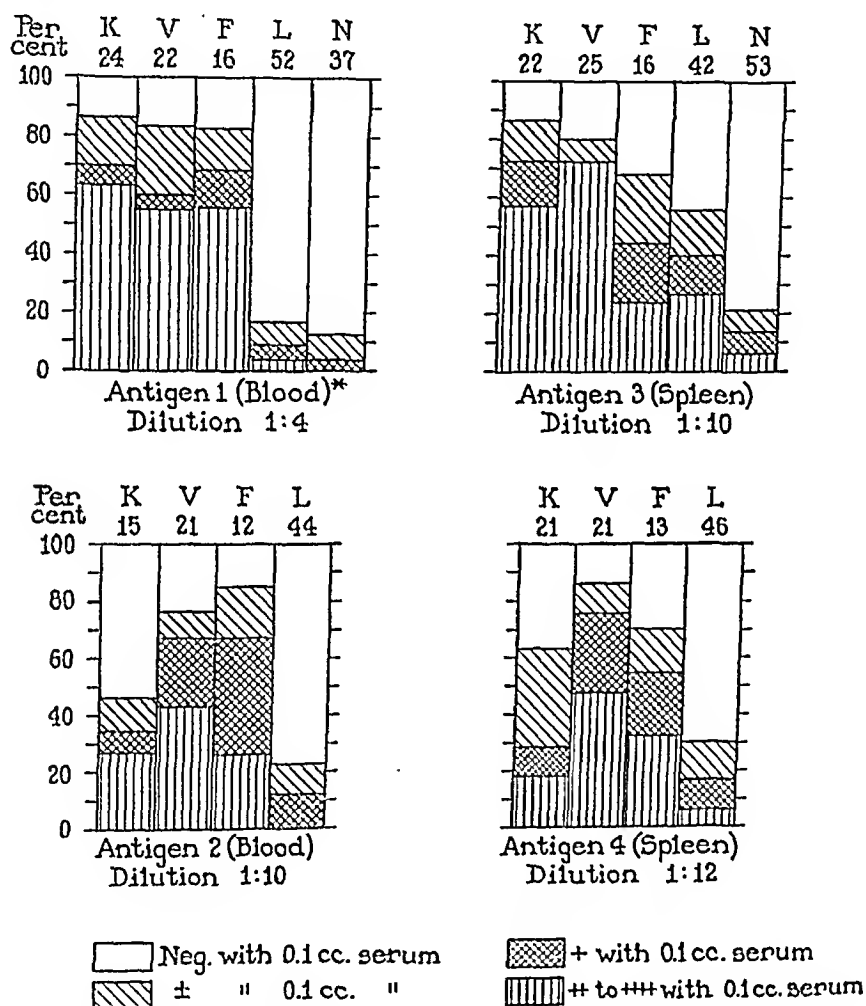
FIG. 1. Specificity and sensitivity of four malarial antigens in complement fixation test with human sera. Letters and numbers at heads of columns indicate kind and number of sera tested. K, sera from *P. knowlesi* infection and lues; V, sera from *P. vivax* infection and lues; F, sera from *P. falciparum* infection and lues; L, luetic sera, no malaria; N, normal sera.

centage of strongly positive reactions. This superiority of antigen 1 was also evident before the results were corrected for reaction with normal monkey cells (see Table II).

Sera from patients without syphilis but infected with *P. vivax*, *P. falciparum*, or both, were tested with antigens 1 and 3. Of the six sera tested with antigen 1, $++$ to $++++$ reactions were obtained with four sera, one was negative, and one gave doubtful reactions with both antigen 1 and antigen N. Of the nine sera tested with antigen 3, reactions with three sera were $++$ to $++++$, with two were $+$,
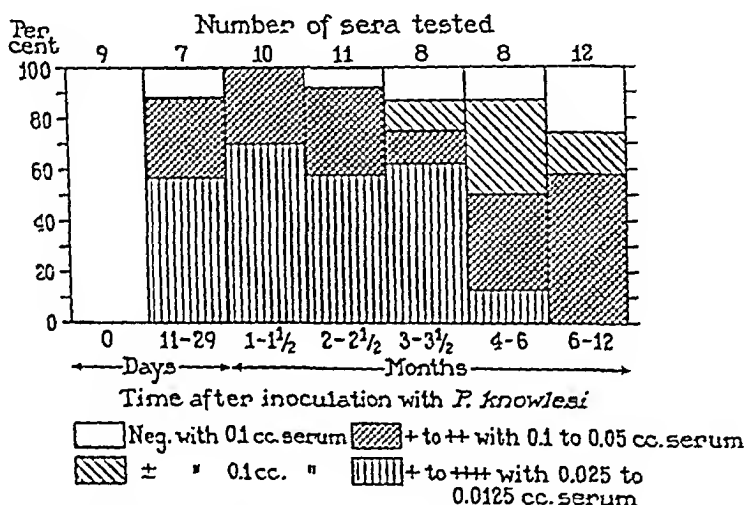
Fig. 2. Complement fixation reactions with sera collected at various intervals after infection with *P. knowlesi*.

and four sera were negative. This series is too small to draw any conclusions except that definite complement fixation may be obtained with sera from patients having malaria alone.

Duration of Positive Complement Fixation after Infection with Plasmodium knowlesi

The results of complement fixation tests with the sera of the twenty-four patients with *P. knowlesi* infection tested with antigen 1, as shown in Fig. 1, and, in addition to these, sera taken from six other patients at various intervals after infection, have been arranged according to time in Fig. 2. Nine sera tested at the time of inoculation

gave completely negative results. The percentage of positive re-
actions reaches a maximum about 1 to $1\frac{1}{2}$ months after inoculation
when all of the ten sera tested were positive, and seven of these gave
strong reactions in amounts less than 0.05 cc. After 4 months the
number of strongly positive reactions diminishes rapidly and most of

TABLE V

*Tests for Plasmodium knowlesi Infection by Inoculation of Monkeys with Blood and
by Complement Fixation with Serum*

Patient	Time after infection	Result of inoculating monkey	Complement fixation test
	mos.		
Bf	1	+	++
Gt	2.5	0	−
Wi	2.5	0	++++
Lx	2.5	0	+++
Jn	2.5	0	+++
Dl	2.5	0	+
Rl	3	0	++++
Pc	3.5	0	++++
Fz	4	+	+++
Ws	4	0	±
Sn	4.5	0	±
Pj	7	0	−
Pc	7	0	++
Lo	7	0	±
Fo	8	0	−
Wk	9	0	+
Sr	9	0	−
Lo	11	0	−
Ce	11	0	+
Pn	12	0	+++

+ = fatal infection with *P. knowlesi.*
0 = no infection.

the results are moderately positive or doubtful. No strong reactions
with less than 0.05 cc. of serum were obtained after 6 months.

Studies on the duration of *P. knowlesi* infections in man (6) have
revealed that blood smears, after being positive for about 1 week,
usually become negative 12 to 31 days after inoculation and remain
negative after this. Parasitological and clinical relapses have been
reported (7). For some time after the blood smear has become nega-
tive, inoculation of 5 cc. of blood into a monkey will produce an acute

infection with *P. knowlesi*. This indicates that the infection in man passes into a latent phase, detectable only by subinoculation into monkeys, which may last as long as 5 months. Twenty samples of blood were taken from patients at various times after acute infection and tested simultaneously by monkey inoculation and by complement fixation. The results are shown in Table V.[2] Only two of the bloods were positive both by monkey inoculation and by complement fixation. Of the eighteen which were negative by monkey inoculation, five were also negative by complement fixation, three gave doubtful reactions, and ten were positive to various degrees. Two patients, Pc and Lo, were tested at two different times after infection.

Although the results of Milam and Coggeshall (6) with monkey inoculations were obtained on a different series of patients, a summary of these results in comparison with the results of the present work on complement fixation is of some interest. Of twenty-seven patients tested at 1 to $1\frac{1}{2}$ months for *P. knowlesi* infection by subinoculation of their blood into monkeys, 60 per cent were positive. This compares with 100 per cent positive reactions in the complement fixation test at 1 to $1\frac{1}{2}$ months (Fig. 2). At 2 to $2\frac{1}{2}$ months thirteen patients were tested by monkey inoculation and 23 per cent were positive, compared with 90 per cent positive in the series tested by complement fixation. Similarly, at 3 to $3\frac{1}{2}$ months 17 per cent of twelve patients were positive by monkey inoculation and 74 per cent by complement fixation; and at 4 to 12 months 10 per cent of ten patients were positive by monkey inoculation, compared with about 50 per cent positive by complement fixation. Although the data presented here are incomplete, they indicate that the complement fixation reaction may remain positive for some time after the presence of infection can no longer be detected by inoculation of the blood into monkeys.

Changes in the Titer of Complement-Fixing Antibodies during Infection with Plasmodium knowlesi or Plasmodium vivax

The changes in antibody titer in the sera of six patients during the course of acute infection with *P. knowlesi* are shown in Fig. 3. The

[2] The series of complement fixation tests represents part of those shown in Fig. 2.
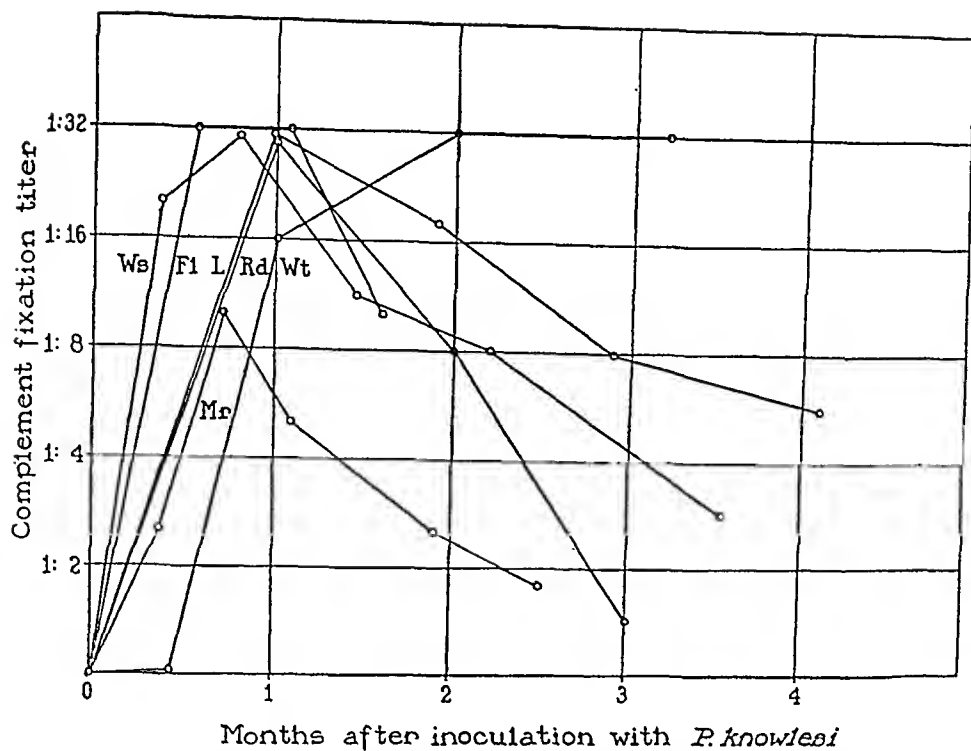
FIG. 3. Changes in complement fixation titer of six human sera before, during, and after acute infection with *P. knowlesi*.
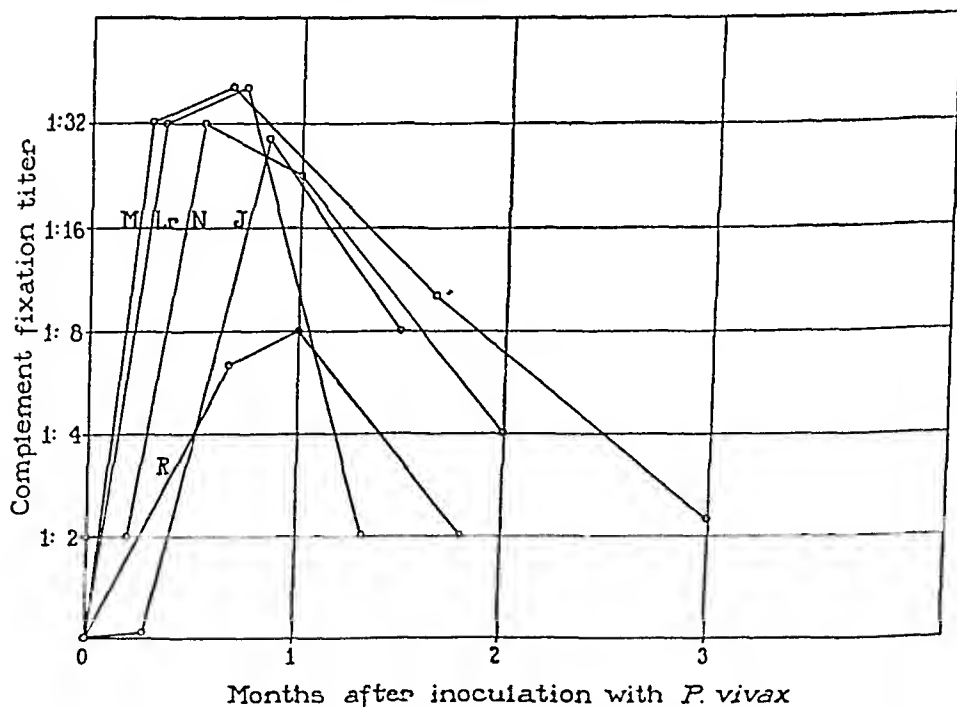
FIG. 4. Changes in complement fixation titer of five human sera before and during infection with *P. vivax*.

complement fixation tests were done with antigen 1, all being set up simultaneously. Controls with antigen N were run on each of the samples of sera. In some cases weakly positive or doubtful reactions were obtained with the normal blood antigen when the titer of complement-fixing antibodies for the malarial antigen was at a maximum. Corrections were made as described in a previous section of this paper. In no case did the titer against the normal antigen go above 1:2.

The titer of complement-fixing antibodies reached a maximum in four of the sera between 12 and 30 days after inoculation. In one patient the reaction was negative 15 days after inoculation, and the titer did not reach a maximum until 2 months had elapsed. It will be noted that the titer tends to fall off rather rapidly in most cases after subsidence of the acute infection. This differs from the results with monkeys (1) in which the infection passes into a prolonged chronic phase with frequent relapses and the complement fixation titer tends to remain at a relatively constant level.

Similar results were obtained with the sera of five patients infected with *P. vivax* in complement fixation tests with the *P. knowlesi* antigen (antigen 1). From the results shown in Fig. 4 it may be seen that the rise in titer is comparable to that resulting from *P. knowlesi* infection, but the subsequent fall in titer appears to be somewhat more rapid after the acute infection with *P. vivax*. This may be due in part to the fact that the patients receiving *P. vivax* were treated with quinine after ten or more paroxysms, while those with *P. knowlesi* usually recovered without quinine.

Effect of Absorbing Human Sera with Normal Monkey Erythrocytes on the Complement Fixation Test for Malaria

If the fixation of complement by human malarial sera and antigens from parasitized monkey cells were due to some constituent of the red cells, and if the control tests with antigen N failed to reveal the antibodies concerned, then, possibly, absorption of the malarial sera with normal monkey erythrocytes might remove the reacting substances. Absorptions were done by the usual method on fifteen sera from patients with *P. knowlesi* or *P. vivax* infection, using 0.5 cc. of packed cells to 1.0 cc. of serum diluted 1:2. The absorption was

repeated once. In some cases the monkey erythrocytes were agglutinated by the sera during the first absorption, but in no case during the second. The absorbed and unabsorbed sera were then tested with malarial antigens from blood and spleen.

Usually the absorption had no effect on the complement fixation with malarial antigen, the titers being undiminished. Examples in

TABLE VI

Effect of Absorption with Normal Monkey Erythrocytes on Complement Fixation with Human Malarial Sera and Plasmodium knowlesi Antigens

Patient	Infecting parasite	Antigen No.	Treatment of sera	Dilutions of serum		
				1:2	1:4	1:8
Fz	*P. knowlesi*	1 (blood)	Unabsorbed	++	+	±
"	"	" "	Absorbed	++	±	—
Wt	"	" "	Unabsorbed	++++	++++	+
"	"	" "	Absorbed	++++	++	±
"	"	4 (spleen)	Unabsorbed	+	—	—
"	"	" "	Absorbed	±	—	—
Lc	"	1 (blood)	Unabsorbed	++++	+++	±
"	"	" "	Absorbed	++++	+++	—
"	"	4 (spleen)	Unabsorbed	+	—	—
"	"	" "	Absorbed	—	—	—
Ir	*P. vivax*	1 (blood)	Unabsorbed	++++	+++	+
"	"	" "	Absorbed	+++	++	—
"	"	4 (spleen)	Unabsorbed	++++	+++	—
"	"	" "	Absorbed	++++	+	—
Mz	"	1 (blood)	Unabsorbed	+	±	—
"	"	" "	Absorbed	+	—	—
"	"	4 (spleen)	Unabsorbed	±	—	—
"	"	" "	Absorbed	±	—	—
Al	"	1 (blood)	Unabsorbed	++	+	—
"	"	" "	Absorbed	++	±	—
"	"	4 (spleen)	Unabsorbed	+++	++	±
"	"	" "	Absorbed	+++	++	—

which slight effects were noted are presented in Table VI. It will be seen that even with sera giving weak complement fixation, such as Fz, Mz, and Al, the effect of absorption with normal monkey cells was negligible. With one serum, Lc, the reaction with spleen antigen was changed from positive to negative by absorption. With one *P. vivax* serum, Ir, there was a moderate reduction in titer.

In the complement fixation test a positive reaction between any serum and a complex material prepared from blood or spleen is a summation of the reactions between several antigens and their corresponding antibodies. In this work we have endeavored to analyze all the possible reactions which occur when an antigen, prepared from the red cells or spleen of a monkey infected with *P. knowlesi*, reacts with the serum of a human being with malaria and syphilis.

With luetic sera from patients having no malaria, three of the four malarial antigens tested gave no higher percentage of positive reactions with Wassermann-positive than with Wassermann-negative sera, and antigen 1, prepared from parasitized red cells, was not more reactive with luetic sera than with normal sera (Table II). When the reactions of normal and luetic sera with antigens prepared from parasitized red cells (antigen 1) and normal red cells (antigen N) are compared, it is found that the malarial antigen gives a slightly higher percentage of positive reactions with both normal and luetic sera, but the differences in reactivity of antigen 1 and antigen N with these sera are not great enough to be very significant. Furthermore, the studies of Kingsbury (3) and our own investigations (1) have shown that the malarial antigen cannot be prepared in the same way as the Wassermann antigen, namely, by alcoholic extraction of organs. The malarial antigen appears to be a water-soluble protein, whereas the Wassermann antigen is probably a lipoid. Thus, the malarial antigen has been shown to be distinct both chemically and serologically from the Wassermann antigen. Certain preparations may contain both the Wassermann antigen and the malarial antigen. For example, antigen 3, prepared from malarial spleen, gave a much higher percentage of positive complement fixation with Wassermann-positive sera than with Wassermann-negative or normal sera.

Certain human sera contain hetero-agglutinins for normal monkey cells, and these antibodies also give a weak complement fixation reaction with extracts of frozen and thawed monkey erythrocytes. The results shown in Table III suggest that these hetero-antibodies are increased during malarial infection, but the increase in hetero-antibodies is not nearly so great as the increase in immune bodies which fix complement with the malarial antigen. Absorption of

malarial sera with normal monkey erythrocytes reduces the complement fixation titer against malarial antigen from parasitized blood only slightly or not at all.   This indicates that most of the fixation of complement obtained with malarial sera is due to malarial antigen-antibody reactions and not to reactions of hetero-antibodies with constituents of the erythrocytes.

The discovery that antigen prepared from *P. knowlesi*, a natural parasite of certain Java monkeys, fixes complement with sera from human beings infected with *P. vivax* and *P. falciparum* is analogous to the results of others (3, 10) who found that antigens from *P. vivax* gave cross reactions with *falciparum* antisera and *vice versa*.   The protective and agglutinative antibodies against *P. knowlesi* seem to be species-specific (2, 11).   There is also considerable evidence that general resistance to superinfection with malarial parasites in birds (12), monkeys (13), and man (14) is species-specific or even strain-specific.   In contrast, the complement fixation reaction for malaria is group-specific.   The possibility that this cross reaction may extend to other protozoal infections of man and animals has not yet been investigated.

With *P. knowlesi* infections in man, the duration of positive complement fixation seems to be greater than the duration of the active infection as determined by blood smears or by subinoculation of the blood into *rhesus* monkeys.   In man, immunity to superinfection with *P. knowlesi* persists for a year or more, which may or may not be due to continued presence of parasites in the body.   The infection in man, however, rapidly passes into a latent phase with corresponding diminution in the titer of complement-fixing antibodies, while in monkeys the repeated relapses stimulate the production of complement-fixing antibodies so that the titer is maintained.   In natural *P. vivax* or *P. falciparum* infections in man the disease runs a course similar to that of *P. knowlesi* in monkeys.   Most of the cases of *P. vivax* or *P. falciparum* infections studied by complement fixation in the course of the present work were treated with quinine so that relapses did not occur.   It is possible, however, that relapses in human malaria produce a rise in the complement fixation titer similar to those observed in monkey malaria.   The value of the complement fixation test as a diagnostic aid in malaria can be determined only after an extensive

study of the reactions of sera from human beings with known malaria in places where the disease is endemic.

SUMMARY

In the studies of complement fixation described in this paper, the antigens were prepared from (*a*) normal monkey red cells, (*b*) parasitized red cells of monkeys dying with *Plasmodium knowlesi* infection, (*c*) the spleens of monkeys dying with *Plasmodium knowlesi* infection; the sera came from (*a*) normal human beings, (*b*) patients with syphilis, (*c*) patients with paresis who were receiving malaria therapy with *Plasmodium knowlesi*, *Plasmodium vivax*, or *Plasmodium falciparum*, and (*d*) patients with malaria alone.

The malarial antigens gave negative complement fixation reactions with 70 to 80 per cent of the luetic and normal sera and weak or doubtful reactions with the remaining 20 to 30 per cent. With the exception of one antigen prepared from spleen, there was no evidence that the malarial antigens were more reactive with Wassermann-positive than with Wassermann-negative sera.

Some human sera give weak complement fixation with antigens prepared from normal monkey erythrocytes, and the percentage of these positive reactions is slightly higher with malarial sera than with normal or luetic sera.

The most sensitive and specific malarial antigen was prepared from dried parasitized red cells by extraction with saline, freezing, and thawing. This *P. knowlesi* antigen gives strong complement fixation with malarial sera from human beings infected with *P. knowlesi*, *P. vivax*, or *P. falciparum*.

The titer of complement-fixing antibodies reaches a maximum about 1 month after the beginning of the acute infection. At this time all of the *P. knowlesi* sera tested were positive. After 4 months the reaction diminishes rapidly in titer but may remain positive for 12 months or longer. With *P. knowlesi* infections in man, the complement fixation reaction remains positive for some time after the infection has apparently disappeared as judged by daily smears and inoculation of monkeys with the blood.

The complement fixation reaction in malaria is group-specific rather than species-specific. Sera from patients infected with *P. vivax* or

P. falciparum react in the same way with the *P. knowlesi* antigen a
the homologous sera.

Absorption of malarial human sera with normal monkey erythro
cytes does not remove the immune bodies which fix complement wit
malarial antigens.

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A COMPARISON OF VIRUS-INDUCED RABBIT TUMORS WITH THE TUMORS OF UNKNOWN CAUSE ELICITED BY TARRING

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The agents called "carcinogenic" act by inducing a protracted tissue disturbance, on the basis of which discrete tumors of unknown cause arise. We have encountered many such growths during experiments on the action of tar to alter rabbit skin in such a way that cancers develop (1) after infection of it with a virus which causes only benign papillomas ordinarily (2). A knowledge of the tar tumors proved essential to appraisal of this phenomenon and hence their study was undertaken. It disclosed the fact that in rabbits such as we employed the benign tumors elicited by tar were of two sorts only, and these so definite in type as to imply the action of specific causes. The commonest sort were papillomas differing distinctively from virus papillomas in certain cytological details but closely resembling them in cellular derivation, mode of development, general morphology, behavior, and fate. The results of a point by point comparison made it plain that the unknown cause of the tar papillomas had neoplastic effects like those due to an authentic virus. The present paper is concerned with all of these subjects.

Material and Methods

Our rabbits, procured from New York dealers, were hybrids of the common, brown-gray (agouti) type. The tar came from the Ostergasfabrik of Amsterdam, and was the generous gift of Dr. Landsteiner. It was smeared on the inside of the ears twice a week, for from 2 to 4 months in most instances, with stripping of the accumulated layer at every third tarring. Over one hundred animals were tarred, mostly as controls in experiments with virus, and some were kept for many later months and sometimes tarred again.

Occasionally the tarring caused tumors to appear within the first month, and they did so within 3 months in more than half of the animals. They were studied by preference soon after they arose, when only a few millimeters broad, before they had undergone the secondary alterations in structure that are frequent when tarring is kept up. Numerous biopsies were made, with a sharp cork-borer. All the material was fixed at once in acid Zenker solution, since in specimens from animals dead only a few hours the finer cytological details underwent change. The stains were methylene blue and eosin.

The Kinds of Tar Tumors

Tar was the first substance utilized in purposeful carcinogenesis, and it was applied to rabbits; but attention soon shifted to mice as a more favorable material. The large literature has been several times reviewed (3), and we have recently summed up the gross effects of the tar employed in our animals (1). It caused benign tumors early and frequently, yet gave rise to cancer only after many months and then but seldom.

Microscopically the tumors fell into four well defined categories:

1. Common Papillomas.—Yamagiwa and Ichikawa (4) termed these growths stalked and broad-based folliculo-adenomas, and noted that the latter were the more aggressive, with carcinomas sometimes originating from them. Both sorts were very frequent in our animals (Table I); and they were found to have essentially the same cytological characters and to grade into each other. They were made up of stratified squamous epithelium, thick and obviously abnormal yet keratinizing in an orderly way, supported upon narrow connective tissue cores or fingers, which occasionally branched (Figs. 5, 7, 40). They first appeared as small, subepidermal mounds which later became discrete, superficial growths of conical, cauliflower, or onion shape, or else,—when maceration had occurred beneath the tar,—raw, fimbriated discs or hassocks. The keratinized epithelium was tenacious, often building high. When tarring was kept up the aggressive growths extended sideways and often downwards in blunt epithelial foldings and tongues, with result that they had a broad base. The shape of indolent ones depended on the changes occurring in the underlying connective tissue. When it proliferated greatly they sometimes became tangential, fleshy spheres, while through its secondary contraction they often became constricted at the base (Fig. 1) or stalked or pedunculated. Persisting broad-based growths eventually underwent similar changes, save in the occasional instances in which they became malignant. Papillomas that had been many times tarred often reached a diameter of several centimeters, and then as a rule they consisted almost entirely of connective tissue. Sometimes their weight gradually pulled them out into pendulum form after tarring was left off, and not a few were torn away while others contracted into mere tabs as result of sclerosis.

If tarring was left off soon after the papillomas appeared, most of them vanished rapidly. Those that were left dried down, often to mere scabs until reconstituted from beneath, and many lost the fleshy character, becoming horns or dry cauliflowers. They tended to persist in proportion to the general changes induced in the skin, and when these had been great, new papillomas often arose. If tarring had been kept up for many months, until they were numerous and crowded, maceration and bacterial infection often persisted later, furthering their enlargement, and occasionally the aural orifice became blocked with pultaceous matter and the animal died of sepsis. Large papillomas tended to persist because their core of sclerotic connective tissue could not be resolved; but sometimes their papillomatous epithelium was ultimately replaced by smooth, non-neoplastic epidermis.

TABLE I

Relative Frequency of the Benign Tar Tumors: Dependence of the Carcinoid Form upon Tarring

Group	Number of rabbits	Period of tarring	Further period after tarring	Papillomas	Transitional growths	Apparent carcinomas (carcinoids)	Frill horns	Total growths
		days	*days*	*number*	*number*	*number*	*number*	*number*
A	18	54–118	2–40	50	10	64	5	129
B	18	61–121	57–259	93	2	0	13	108

The growths tabulated came from groups of animals tarred for the same general length of time, but differing in that the tumors were not procured from one group until long after tarring had been stopped. Only growths which were examined microscopically are listed, and in some animals they formed but a small proportion of the total number. The basis of the classification is given in the text. The frill horns could be readily identified in the gross, and every one seen was taken for section. Carcinoids also were often recognizable as such, and a greater proportion of them were examined than of papillomas. It follows that frill horns were actually much rarer than the figures indicate, and carcinoids somewhat less common.

The papillomas have often been pictured. The several hundred that we have studied microscopically differed not a little in general pattern. Some were completely superficial and regular, with widely spaced papillae and dry keratin between; others had many crowded peaks; others extended down in broad tongues that keratinized secondarily, often becoming cystic; while others still broke up along the base into what appeared to be anaplastic, squamous cell carcinomas. Yet despite this diversity the cells of all, so long as they remained benign papillomas, manifested identical *qualitative* alterations (Figs. 33 and 35), and whenever differentiation and keratinization took place it was in the same way. The minute histological findings will be described further on.

2. Frill Horns.—Though possessed of a distinctive morphology, these growths have not heretofore been reported. They were seldom more than 3 mm. across at the base, yet were readily recognizable in the gross because they built up into narrow, cylindrical or strap-shaped, curved or twisted horns (Figs. 8, 9), often 2 to 4 cm. high, of the same diameter everywhere, usually cream-colored but sometimes light or dark gray, and always dry nearly to the skin level. Here was a slightly bulging, fleshy collar covered with epidermis stretched smooth.

The frill horns arose at the same time as the papillomas, but were infrequent (Table I). Usually there were several when any. Though appearing wholly superficial, they burrowed slowly, and ineffectually, their epithelium extending downwards in a jagged, crescentic frill (Figs. 10, 11), which advanced so gradually as to be effectively barred from the cartilage by reactive connective tissue proliferation. Very occasionally a fold in the frill mimicked a papillomatous finger. Its living layer was not so thick as in papillomas, and stained deeper with methylene blue. The individual cells were irregularly polygonal, with birdseye nuclei, and they became larger when differentiating, and keratinized abruptly without preliminary granulation or flattening (Fig. 12). The keratinized material stained pink or red with the eosin and methylene blue stain, whereas that of tar and virus papillomas failed to color or took on a faint blue; and the nuclei of the cells newly dead stippled the keratinized material with dark blue, oval dots, which faded later. In the gross the horns were close-textured, horizontally laminated, sometimes with an ill-defined, vertical striation, and microscopically their keratinized material appeared dense, showing none of the loose, reticular pattern, indicative of old cell boundaries, which is visible in papillomas. The connective tissue underneath the growths, like that under papillomas, showed only chronic inflammatory changes, with a scattering of macrophages and lymphocytes. Continued tarring had little effect to render the epithelium disorderly, and when it was discontinued the horn nearly always went on growing, unlike most papillomas, and sometimes new ones appeared (compare frequency in groups A and B of Table I). Retrogression was noted only once.

3. Carcinoids.—A large proportion of the tumors appearing early had the morphology of squamous cell carcinomas (Figs. 13, 14, 16, 18), yet when tarring was left off they vanished or assumed a benign form (Figs. 17, 19). These were the growths generally termed carcinoids (5). Often they were markedly invasive at first (Fig. 15), their cells straggling down individually, or penetrating in narrow strands through a reactive connective tissue which was sometimes mucoid like that elicited by many squamous cell carcinomas; and not infrequently they extended through lacunae in the cartilage to the outside of the ear, forming mounds there which ulcerated. They could usually be recognized in the gross, being raised, raw, ragged discs with *infiltrating edges* (though papillomas occasionally assume this form), or else raised, discoid ulcers as much as 1.5 to 2 cm. in diameter. Yet even when proliferating within lymphatics (Figs. 15, 18) they never metastasized; and though tarring was kept up they did not continue to grow destructively but eventually took on gross forms like those of old papillomas, or else retrogressed.

Repeated biopsies showed that the supporting connective tissue had proliferated actively, walling off the growths, and that in proportion as this happened their epithelium ceased to invade, underwent differentiation, and in most instances became papillomatous (Figs. 16, 17), though cell islands of carcinomatous aspect occasionally persisted. Frequently the deeper epithelium rounded up into cysts filled with keratinized material (Figs. 18, 19) and lined with a stratified squamous layer devoid of neoplastic features; but in other instances all the deep cells keratinized, died, and were eventually resorbed. Those carcinoids which disappeared did so in these ways, save in an unique instance of a growth dying *en masse* without differentiation, as if from some intercurrent malady. Carcinoids which appeared early, before the tarring had effected great cutaneous changes, generally vanished soon after it was left off, whereas when the skin had undergone much alteration they often persisted, either as papillomas or cysts.

The earlier observers, conceiving that the carcinoids were cancers, followed with intense interest the various retrogressive changes here described (6), and these have been many times figured. Some workers attributed them to the associated connective tissue alterations whereas others held that this could not explain them.

The papillomas of animals tarred 2 to 4 months not infrequently broke up along the base into what looked to be carcinomas (Table I, Transitional Growths). Yet when tarring was left off these all underwent one or another of the changes just described. The figures of Table I show that a wholesale disappearance of carcinoids took place then. Amongst 129 growths of 18 rabbits, examined 2 to 40 days after the last tarring, 64 had the morphology of squamous cell carcinomas and 10 were transitional growths. Among 108 tumors procured 61 to 121 days after the last tarring of 18 comparable animals, there was not a single apparent carcinoma and only 2 transitional growths. All the rest were either papillomas or frill horns. Keratinized cysts have not been included in the figures.

To determine whether the carcinoids would grow in a new situation, pieces of 20 that were big and actively enlarging were punched out, hashed separately in Tyrode solution, and implanted in the voluntary muscle or connective tissue of he axilla or groin of the host. Here they behaved as did bits of 19 tar papillomas treated in the same way, their cells either differentiating, dying, and disappearing without sign of growth, or rounding up into pearls lined with stratified quamous epithelium (7). Similar results had been reported by Ferrero (8). lair follicle epithelium, sebaceous glands, and bits of cartilage, accidentally introduced with the implants, survived in the new situations.

4. *Carcinomas.*—In the early work on the tar cancers of rabbits (4, 6) carcinoids ere frequently taken for carcinomas. Recent authors agree that usually the latter evelop only after tarring has been done for a long time. Guldberg (9) elicited pillomas after 61 to 140 days in all of 23 rabbits, but carcinomas appeared in ly 7 and not until the 276th day of tarring at the earliest, and in most cases at out the 330th day. Our tar produced nearly always a fatal toxemia or liver rhosis, when applied regularly for more than a few months, not sufficiently g to elicit cancers. These developed, however, in 2 animals tarred again after

an interval. Tarring of one of them had been done throughout periods of 5½ and 6 months, the cancer appearing after 21 months in all. The first tarring of the other was for 4 months, with later applications for periods of 4 weeks and 3 weeks. Between the 20th and 22nd months 2 cancers appeared. In both rabbits metastasis took place to a regional lymph node.

Most tar cancers of rabbit skin are squamous cell carcinomas arising secondarily from papillomas (4, 6). The 3 we have studied had this derivation, and the metastasis of one was cystic and exhibited the papillomatous form (Fig. 20). The literature reports occasional trichoepitheliomas and carcinomas originating from sebaceous glands, as also spindle cell and polymorphous cell sarcomas.

Effects of Tarring the Tumors

In a previous paper we have dealt with the influence of continued tarring upon the morphology and behavior of virus-induced growths (10). It has even more pronounced effects on many of the tar tumors, and is often the determining factor in their fate.

Tarring causes acute inflammation of the skin, with chronic changes later if the applications are kept up (3). In some of our animals the ears were thick and hot when tumors first appeared, and not infrequently a foul maceration had taken place under the tar with result that the growths were exposed to bacterial infection, while additional opportunities for this were provided by trauma incidental to the repeated stripping of the tar. The conditions were such as are known to further the growth of tumors generally. In the loose, proliferating connective tissue most of the carcinoids grew fast and invasively; and it was frequently edematous, not only beneath them but sometimes opposite them on the outer side of the ear (Fig. 47). Thither they often extended through lacunae in the cartilaginous plate, sometimes by way of the large lymphatics. Ferrero (11) has noticed that connective tissue disturbances favor carcinoid activity. The papillomas also tended to grow rapidly and irregularly, where the connective tissue was most disturbed, and sometimes they became anaplastic along the base. The frill horns, on the other hand, proved unresponsive to stimulation, as has already been stated.

The continuance of tarring notably stimulated the activity of papillomas and carcinoids, and leaving it off had pronounced adverse effects upon them. Unless it had been long kept up the skin at once began to revert to the normal, the acute inflammation and edema disappearing, the circulation lessening, the connective tissue sclerosing, the epithelial proliferation slowing, and the epidermal surface drying down and desquamating. The tar tumors dried down too, and most of the papillomas disappeared, while the carcinoids vanished as well, or became papillomas or cysts. The more rapidly the skin returned to the normal the more sweeping was the disappearance of growths. In occasional animals tarring called forth tumors very early, in 4 to 5 weeks (Fig. 18), and sometimes then the ears

were studded with them, many being anaplastic carcinoids as biopsies proved; yet if no more tarring was done all disappeared within a fortnight. In these cases the ears had undergone but slight general changes and rapidly resumed the normal appearance.

Very different was the outcome when tarring had been kept up 3 to 4 months or had been done throughout several periods. The ears then remained thickened and indurated for many weeks, and scurfy layers formed and reformed on them, attesting to persistent epithelial activity. That the other tissues were also in an abnormally excitable state was shown by the rapid and complete filling in of holes 3 to 10 mm. across which had resulted from punch biopsies. Even though the ears presented a normal aspect when the holes were made these closed more quickly than usual, sometimes with cartilaginous thickening, while when the skin had remained thickened and scurfy not only was healing notably rapid but there was often a lumpy overgrowth of cartilage. The conditions thus disclosed were obviously favorable to the tumors, as shown by their frequent persistence and increasing disorder, and by the appearance in some instances of new ones. They proved most likely to persist along the edges and near the tips of the ears where trauma acted to prolong the abnormal state of affairs (Fig. 1); and foci of inflammation beneath them or in their stroma frequently aided their course and acted to distort their form. Some of these findings are not new, yet all deserve emphasis because they bear upon the riddles presented by the occurrence and disappearance of the carcinoids, and the eventual change of papillomas to carcinomas.

The Place of the Carcinoids as Tumors

The carcinoids arose at the same time as the papillomas, and exhibited a like tendency to disappear after tarring had been left off. They often derived from papillomas and those persisting frequently became such, and were then indistinguishable from growths primarily papillomatous. Some of the carcinoids however retrogressed to keratinized cysts, as rarely happened with papillomas; but this was only because the latter did not penetrate deep. When they did so, or were implanted within the host, the cystic form was frequently assumed; and the cysts differed in no wise from those originating from carcinoids. There was obviously a close relationship between the two sorts of tumors. In seeking to understand it certain observations on virus papillomas come to mind. These growths often assume a malignant appearance if experimentally stimulated or implanted in notably favorable locations, especially if inflammation of bacterial origin ensues (12); and yet they are not true cancers, for they revert at length to the papillomatous state. We have termed the phenomenon *factitious malignancy*. Everything that is known of the tar carcinoids indicates that they are expressions of a factitious malignancy of growths which have intrinsically the nature of tar papillomas. Their complete dependence upon tarring for their cancerous form and behavior becomes comprehensible in this light, as does also their frequent derivation from papillomas and transformation into them, or into keratinized cysts such as papillomas may secondarily form.

Many carcinoids have a malignant aspect from the first (Fig. 13). Becoming visible as small, subepidermal mounds, they grow down from an intact surface epidermis or from the epithelial cells of hair follicles; and if tarring is stopped they may die by differentiation and keratinization, and be resorbed without giving sign of any change to papillomas. The question arises of whether growths of this sort, and those carcinoids of secondary origin which disappear in the same way, do not differ intrinsically from the ones which undergo conversion into papillomas or cysts. The answer is supplied by the course of events when tarring is kept up. Then the great majority of the carcinoids keep on proliferating, though only to assume the state of papillomas when tarring is eventually left off (group A, Table I), or, if disappearing, to undergo the same changes as these. It follows that such carcinoids as die by keratinization do not differ from the rest save in having even less ability to maintain themselves.

All in all the conclusion seems justified that the carcinoids owe their malignant aspect and behavior to the effect of extraneous conditions upon growths having an intrinsic character which ordinarily finds expression in benign papillomas.

The greater the acute inflammation of ears on which tar has evoked tumors the greater was the proportion of growths exhibiting the carcinoid form. Sometimes all showed it for the time being. Yet frequently two tumors situated side by side, exposed that is to say to much the same local conditions, were the one a carcinoid, the other a papilloma (Fig. 24). The inference seems justified that papillomas differ much in their responsiveness to those local influences which result in spurious malignancy.

It has been frequently assumed that the carcinoids are carcinomas brought into being and maintained by tarring, and devoid of the power to grow independently. Carcinoids differ, however, from true carcinomas, not alone in their inability to proliferate independently as such, but in a more significant feature, namely the reversible malignancy of their cells. Real squamous cell carcinomas of man have been known to retrogress on rare occasion,—and they do so by differentiating and keratinizing completely (13), as happens with many carcinoids,—but they never, like the latter, undergo conversion into benign growths. Carcinomas they remain until the last.

A few of the small tar tumors procured from animals which survived long tarring, or which were tarred throughout several periods, were found to have the microscopic aspect of cancers, although tarring had been discontinued for many months. The general state of the ears was notably pathological, and one may assume it to have given unusual encouragement to the persistence of carcinoids; yet the development of true cancer, if only in three instances, indicates that the apparent malignancy of some of the small growths mentioned may have been real.

Comparison of the Tar Papillomas with the Virus Papillomas

The resemblance of virus papillomas to the tar papillomas has already been reported upon briefly (14). The virus growths studied had been obtained by the

direct inoculation of scarified normal skin, mostly of the animal's side, and in consequence they derived from the surface epithelium, and were subjected to none of the collateral influences that tarring exerts. Now we have studied many papillomas due to the localization of virus out of the blood stream into the skin of ears repeatedly tarred, or acutely inflamed by the application of carbon bisulfide, as also growths resulting from the direct infiltration of normal ears with the inoculum, by way of a vein, with tarring later. Tar has also been applied repeatedly to virus papillomas resulting from tattoo inoculations into normal ears. These diverse materials gave consistent findings. Previous experiments had demonstrated that when skin has been tarred for some weeks prior to infection with virus by way of the circulation a considerable variety of tumors results, some of them cancers (1). For the present comparison only those virus papillomas were utilized which arose under conditions excluding this complication, most of them from ears which had been tarred just enough to bring about localization of the virus, or from ears which were not tarred until after virus infection had taken place.

Many of the facts to be cited of the tar papillomas are truisms, and no reference will be made to the literature substantiating them. The pictures illustrating the comparison generally show growths which had not yet undergone the complications which result from continued tarring and inflammation of the supporting tissues. In other respects they are representative.

Cellular Derivation.—Tar papillomas derive mostly from the epithelium of hair follicles, whereas those produced by virus rubbed into scarified skin all spring from the surface epithelium (2), as just remarked. But if the virus is thrown into the blood stream after one or two tarrings of the ears to insure localization, most of the growths arise precisely where the tar tumors begin, namely at the lower side of the hair follicles (Figs. 21, 22). This is the case also when normal ears are directly infiltrated with virus fluid by way of a marginal vein, and are tarred a few times later to render the inoculum effective (10). As in the case of tar papillomas the growths arising from the follicles usually begin on their basal side, with a proliferation of cells in the stratum germinativum, resulting in the formation of blunt, broad processes ("elephant's feet"), and a folding inwards of the epithelium in the papillomatous pattern.

Morphology.—On the inside of the ear both the tar and virus papillomas soon erupt and become surface growths. On the outside, though, where the skin is not bound tight to the cartilage, they may reach a diameter of 4 to 10 mm. before the stretched, shiny epidermis over them dries or gives way. Here they lie embedded as more or less flattened acorns or spheres (Fig. 18 *b* of reference 15). Most of those due to tarring are creamy or pink, though some are light gray and a few dark; whereas the virus papillomas are much more frequently pigmented, often dark gray or almost black. Both are opaque toward the center, and here they consist of keratinized material formed by a proliferating rind of stratified squamous epithelium of abnormal character (Figs. 23 to 26). This is usually thickest where deepest; and its infoldings are usually somewhat more numerous

and crowded in the virus papillomas (Figs. 27 to 30),—as would follow from their greater vigor. The pattern becomes more complex as the growths enlarge, and the epithelium may thrust irregular tongues downwards (Figs. 29, 30), an invasion encouraged by continued tarring.

After they have erupted the papillomas of both sorts become superficial cones, cylinders, "cauliflowers," or "onions," or they form tall, dry horns. Their keratinized material is tenacious and builds high. They may consist of it practically to the base, or connective tissue processes covered with living epithelium may extend far up into it. The processes branch sparsely (Figs. 4 to 7). As a rule virus papillomas proliferate the more rapidly and hence are plumper, more fleshy, do not dry so far down, or split so frequently into cauliflowers, retaining instead the onion or conical shape (Figs. 2, 3). Yet when tar papillomas grow rapidly, or virus papillomas slowly on the same ear, they cannot be told apart individually in the gross unless they are melanotic, which is never the case with actively enlarging tar papillomas though frequent with those due to virus (15).

Under low magnifications the tar and virus papillomas are nearly alike (Plate 20 et seq.), but they differ distinctively in their finer cytology (Plate 25). In the virus growths the proliferating cells of the stratum germinativum are larger than normal, with abnormally big, vesicular nuclei (Figs. 32, 34). They get larger still as they differentiate, undergo but little flattening in the stratum granulosum, and the granules forming there are usually few, and range from small to very coarse. The cells of the tar tumors on the other hand, though unusually large in the stratum germinativum, do not increase further in size, and they flatten in the stratum granulosum, appearing fusiform or oat-shaped on cross section, with numerous small granules darkening the cytoplasm (Figs. 33, 35). The nuclei of the virus papillomas, big and vesicular from the first, increase markedly in size as differentiation goes on, and the chromatin marginates, whereas in the tar papillomas they alter little, though large primarily, and the chromatin remains central, giving a birdseye appearance. On keratinization the nuclei of the virus growths sometimes become pycnotic for a brief while, in the deep crypts between the papillae (Fig. 32), whereas those of the tar papillomas usually lose almost at once their capacity to stain. The keratinized scales, however, assume the same appearance in both instances, their outlines forming a loose reticulum indicative of some swelling. Ordinarily they fail to stain with eosin and methylene blue though they take the blue if the staining is forced.

The cells responsible for the gray or black hue of many virus papillomas, and of a few of those due to tar, are melanoblasts for the most part (Figs. 34, 35), though the epithelium in the lower part of the proliferating layer often contains brown granules. The melanoblasts are frequently black with pigment granules which tend to be larger, coarser, and darker colored in the virus growths; and such cells accumulate much more slowly in the tar tumors, only coloring those which enlarge very gradually. The exceptional tar papillomas which were almost black had become so in the course of months during which they scarcely changed in size. Gray tar tumors which begin to grow fast soon become pink. The prolifera-

tion of the melanoblasts of virus papillomas, on the other hand, frequently keeps pace with their enlargement, and in consequence they remain sooty throughout months of vigorous proliferation (2). The melanoblasts are no essential constituent of the growths, however, but are included fortuitously (16), and they may sooner or later disappear or be outstripped (17). The same holds true in more striking degree of the tar papillomas, and the melanosis is of essentially the same sort.

Any irritation of the skin of gray-brown rabbits may bring on a graying of skin previously pink, and long-continued tarring often results in black freckles (Fig. 1), due to aggregates in the corium of chromatophores stuffed with pigment. Sometimes the source of this can be traced to a distant tar tumor or patch of epidermis containing active melanoblasts or epithelial cells in which pigment is abundant. Where sooty tar or virus tumors have disappeared a dark patch may persist, due to residual melanin in melanoblasts and chromatophores. Such patches were especially frequent in the case of the virus tumors (Fig. 46), and phagocytes dark with pigment were sometimes present in the lymph nodes into which they drained.

The shape of tar papillomas is markedly affected by continued tarring. Many become pedunculated (Figs. 40, 43), the proliferating connective tissue underneath the growth lifting it away from the ear. This happens less frequently with virus papillomas, because their cells multiply faster, are more aggressive, and hence can maintain their original place near the cartilaginous plate. Yet a pedunculation of virus papillomas may occur, even in the absence of any tarring, if they become indolent (Fig. 42) (12), and it often takes place despite rapid enlargement when tarring is done (Fig. 39). The likeness to pedunculated tar tumors may then be complete at low magnifications (Figs. 39 to 43) as well as in the gross. Virus papillomas on the rabbit's side have never become pedunculated in our experience. There the fibrous corium is a thick, tough sheet which does not yield to the weight even of huge growths, while furthermore none of our animals was tarred in this situation.

The virus papillomas of some rabbits are fleshy, whereas those of others, although doing well, are dry almost to the skin (Figs. 2, 3). This is true also of tar papillomas. When growths of both origins coexist on the same ears they frequently vary together in such respects, host influences obviously affecting them in the same way.

Retrogression.—When tarring has not produced enduring effects on the skin most of the growths due to it dry down, scab, and the scab comes away leaving a smooth scar (1). Some drying down and disappearance take place in any case unless the tumors are crowded and macerating. Most of the papillomas which persist become deeply cleft, brittle cauliflowers, or petaloid excrescences with constricted bases, or else horns or narrow cones, dry nearly to the skin. A few remain fleshy, however, and these usually have an onion shape. Some growths reconstitute themselves from beneath after scabbing. These exhibit no peculiarities, nor do such papillomas as may appear later. Generally virus papillomas are

much less affected by the discontinuance of tarring, as might be expected from their great vigor; and many continue to grow rapidly, and remain of onion shape or broadly conical. Some, however, assume the cauliflower or petaloid form, or become high horns.

The retrogression of tar and virus papillomas is attended by the same microscopic changes. When it is very slow the protruding, finger-like papillae become fewer and narrower, the layer of living epithelium shallower, the underlying connective tissue denser, and eventually the mass consists almost entirely of keratin (Figs. 42, 43). These changes, largely referable to sclerosis of the supporting tissues, are especially frequent in pedunculated growths.

When retrogression is rapid the growths get lower, owing to an unrepaired loss of keratinized material, their papillae shorten, and instead of broad "elephant's feet" of living epithelium along their base one finds narrow, irregular processes, separated and underlain by a reactive connective tissue containing many macrophages and lymphocytes (Figs. 44, 45),—cells almost absent from the slowly dwindling growths just described, and infrequent in tarred skin returning to normal. The epithelial processes appear as if invasive, and mitosis is still going on in their cells (as in retrogressing mouse carcinomas also (18)); yet the growths are actually smaller and more superficial than before, connections with the sebaceous glands and hair follicles have appeared (Fig. 44), and the underlying cellular accumulation (macrophages, lymphocytes) is of the sort which accompanies the retrogression of tumors generally. At length only a smooth scar is left, covered with somewhat thickened squamous epithelium devoid of distinctive peculiarities.

After tar carcinoids and papillomas are implanted in the subcutaneous tissue or leg muscles of the host they either die by keratinization or round up into small cysts, as already stated. The same holds true of virus papillomas implanted in hosts which develop a resistance to these growths (19). In both cases the living epithelium at the periphery of the cysts ceases to have a neoplastic appearance.

Conditions of Origin.—The real cause of the tar papillomas is something other than tar. The virus on the other hand is directly responsible for the papillomas it produces. The unknown causes of the tar tumors in general, and of the growths due to other "carcinogens," act only because of some change of a peculiar nature that these agents slowly bring about in the tissues; whereas the virus, though rendered unusually effective by this change (1), can act upon epidermis which has been merely scarified or acutely injured in other ways, as by an application of sodium bisulfide (10), or by inflammation due to a subcutaneous abscess. In contrast to the causes for the tar tumors the virus needs little help to be pathogenic, and only non-specific help, while the growths it produces need no help whatever in most instances. Nevertheless one may recall, as bearing on the possible causation of tar tumors, that tarring brings about conditions exceptionally favorable to localization of the virus out of the blood stream, that it enables it to engender growths after introduction into normal skin,—when otherwise this would not happen (10),—and that it exerts a stimulating effect upon virus papillomas which

is often pronounced, though not ordinarily decisive for their fate as it so frequently is in the case of tar tumors.

The ears of some rabbits are especially sensitive to tarring; and the more markedly it changes them the more likely are tar tumors to arise. This holds also for the papillomas which result from an intravenous injection of virus, though the conditions making for localization of the virus under such circumstances must be discriminated from those determining the formation of tumors (10). When both ears have been tarred equally, tar tumors appear in approximately the same number on both. So it is also with the papillomas arising after virus has been thrown into the blood stream: the average for both ears is the same unless the circulation to one was interfered with while the virus was circulating (10).

The tumors due to tar are local epithelial phenomena, the outcome of happenings at special points in the hyperplastic epidermis. So also with the virus tumors, though when an immense quantity of virus has suddenly been placed in circulation it may infect the tarred epidermis at so many spots as to give the impression that a generalized neoplastic transformation has occurred. Theoretically one should occur if the virus reached and infected every cell in the basal layer of the stratum germinativum. Actually, however, what appears to be generalized neoplastic change after massive virus infection, is usually due to a secondary coalescence of growths arising at numerous, separate points, as sections taken at early stages have proved.

The increase in size of the papillomas due to virus is due to multiplication of the cells with which it becomes associated primarily, not to contact infection of adjacent elements (12). This holds good also for the tar tumors. Both are frequently multicentric in origin, but the tar tumors only exceptionally arise in hordes. In an unique instance, the ears of a rabbit tarred in the usual way for 110 days were practically covered with tumors, close crowded and becoming confluent. In a few small blocks taken at random 61 papillomas could be discerned, as also 2 carcinoids and 2 frill horns. These findings were not included in Table I, to avoid distortion of the figures. The ears of an animal examined after tarring throughout 13 months showed large expanses of low, diffuse papillomatosis, which might have been interpreted as the result of a generalized change in the epidermis had there not been charts proving it due to a coalescence of growths originally separate and discrete.[1]

After an intravenous injection of virus, hosts of papillomas often arose on the outsides of ears rendered hyperkeratotic by tar transferred from the insides, and on the neck as well, where similar changes had taken place for the same reason.

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[1] Suntzeff, Burns, Moskop, and Loeb, studying the downgrowths of uterine epithelium in mice given an estrogen (*Am. J. Cancer*, 1938, 32, 256), have noted that they often occur at many points, and sometimes everywhere as if by a generalized transformation. Their conclusion that these findings are scarcely compatible with the working of a virus, would seem unwarranted in view of our observations.

The growths were marked as due to the inoculum by their appearance almost simultaneously after the usual incubation period, by their rapid growth and frequent, deep melanosis, by their cytology, and by the almost complete absence of tumors from such situations in animals merely tarred, unless the tarring had been kept up for many months (15).   When this was the case scattered tar papillomas eventually appeared on the neck and outsides of the ears, and they derived from the same structures and took the same forms as the virus papillomas (Figs. 21 to 30).   The earlier appearance there and greater frequency of virus-induced growths were obviously referable to the relatively slight epidermal changes required for the action of the virus, to the abundance of it provided, and to the vigor of the growths it caused.

The tumors due to tarring occur at irregular intervals, and new ones may keep on appearing for months or years afterwards, if the skin has been rendered enduringly pathological.   Nearly all of the growths due to virus, on the other hand, appear within a few weeks of the intravenous injection unless the skin has undergone such change.   Under these circumstances characteristic virus papillomas may continue to appear sporadically for at least 4 months after the inoculation, a period double the longest observed in the case of normal skin.   In one experiment a rabbit's ears were acutely inflamed by swabbing them with barium sulfide, virus was injected intravenously, and 120 days later tarring was begun.   At this time there were 3 papillomas present, which had appeared shortly after the injection, but later, as tarring went on, a fourth developed, shortly before the 160th day.   Like the others it was marked as due to the virus by the retention of a slaty hue despite rapid enlargement, as furthermore by its characteristic cytology.

Course.—Tar papillomas may run widely differing courses though all are markedly responsive to change in the general state of the ears.   Some of the earliest to arise and the swiftest to grow may vanish despite continued tarring, and while others nearby are rapidly enlarging.   Some, beginning late and growing slowly, continue to grow after tarring is left off though all their neighbors are disappearing.   The majority, however, enlarge or retrogress together.   So too do the virus papillomas resulting from a direct inoculation of normal skin; for they are mostly multicentric in origin and hence what they do constitutes an average response.   But when the virus is distributed on the blood stream,—providing, as this does, an opportunity for its individual entities to infect single cells,—and the state of the cells has been altered by preliminary tarrings, the growths may not only appear at widely differing times and vary much in neoplastic character (10), but often certain of those which appear to be typical virus papillomas may grow vigorously while others wholly resembling them and situated on the same ear are retrogressing.   They now differ in incidence and course as do the tar papillomas, though not to nearly so great an extent.   This is scarcely surprising in view of the fact that the virus exerts much more compulsion on the cells than does the cause for the tar papillomas, and is far less dependent on contributory circumstances for its continued action, as expressed in behavior of the growths. What the virus does is of predominating importance for the course of neoplastic

events, once these are under way, not what attendant or intercurrent circumstances do, as in the case of most tar tumors.

The superior pathogenicity of the virus is usually evident in the behavior, as well as the form, of the papillomas engendered by it: they enlarge more swiftly than most tar tumors and appear relatively unaffected when tarring is discontinued. Nevertheless in some animals they may retrogress all at once, a host of growths which have long flourished every one vanishing, perhaps within a few days, even though tarring is kept up. This behavior is due to the development of a generalized host resistance (20), which is not always comprehensive in its effects when the papillomas are situated on tarred ears, some of them continuing to enlarge in contrast to the majority. And even when the resistance is all-inclusive it is often transient, some growths reappearing after a few weeks in their previous positions, marked by patches of pigment in the corium. Tracings were made on cellophane in a few cases while the growths were dwindling, in order to record their exact situations; and after they had vanished tarring was begun anew. Some reappeared, but one cannot be sure that the tarring was responsible since recurrence can take place without it (20). Recently we have found that renewed tarring may call forth tar tumors where they were before, months after seemingly complete disappearance.

Whether any host resistance develops to tar papillomas is uncertain. The wholesale retrogression often witnessed after tarring has been stopped is plainly due in most cases to involutionary changes in the skin of the ears; and the sensitiveness of the tumors to these latter renders it difficult to tell whether other adverse influences are working upon them. Certainly host resistance, if such there be, is much less comprehensive and effective than that provoked by the virus tumors, which is notably selective, frequently causing complete disappearance of virus papillomas while exerting no visible influence upon the tar tumors associated with them.

Spurious Malignancy.—The progressively increasing disturbance of the skin, when tarring is kept up, acts to further the growth of tar papillomas, to disorder their form and to render them aggressive. Some extend deep, forming numerous small cysts secondarily, while the pattern of others becomes irregular and complex. They may penetrate to the outside of the ear and form secondary masses there, even while retaining their original morphology (Figs. 37 and 38). Not a few of them break up along the base as if becoming carcinomas, though actually they are but carcinoids in most instances; and growths of the latter sort keep on appearing. Yet if tarring is stopped after 2 to 4 months all of the apparently malignant tumors take on the form of benign papillomas, or become cystic or disappear. It is plain that tar tumors are very prone to spurious malignancy.

Virus papillomas are much less sensitive to continued tarring, though under its influence they too may become somewhat disorderly, extend down and form cysts, or exhibit complexities of pattern. Often they become very aggressive and grow through to the outside of the ear (Fig. 36). Yet even when proliferating with prodigious rapidity they do not assume the carcinoid form, although it is seen when the growth is implanted in the interior organs, especially under condi-

tions of local inflammation (12).   The virus evidently exercises a stricter forma-
tive influence upon the cells with which it is associated than does the cause of
most tar papillomas.   Yet the formative influence of this unknown cause is
notably strict in not a few instances, the growths remaining papillomas, however
much tarred, and under circumstances which lead others immediately next to
them to be carcinoids (Fig. 24).

So long as tar and virus papillomas continue to be such they retain the cytologi-
cal features distinctive of them, no matter how complicated or disorderly their
pattern; but most of these features only become manifest as differentiation takes
place, and they are lost in proportion as the cells fail to differentiate, as when
they become carcinoids or carcinomas.   Markedly anaplastic growths deriving
from tar and virus papillomas cannot be distinguished as of differing origin.   The
character of the cells in the papillomatous part of the cystic metastasis shown in
Fig. 20 identified the growth as a tar tumor, whereas the cells of its invasive por-
tion yielded no information in this regard.

The Development of Carcinomas.—The course of events when tarring was dis-
continued after a few months proved that all of the apparent malignancy then
existing was spurious; but this was not the case when tarring had been kept up
for a long time or had been done throughout several periods.   In 2 rabbits thus
treated genuine cancers arose, as already stated, while in others malignant changes
seemed under way.   The 3 cancers all derived from papillomas, like the generality
of these growths in rabbits (4, 6).   In the present comparison we are concerned
only with cancers of such derivation.[2]   The changes which take place when tar
papillomas become malignant have often been pictured (4, 6), and they are
morphologically identical with those taking place in virus papillomas which become
malignant, the carcinoma cells stemming from the papilloma cells by alterations
of greater or less magnitude (21).   Furthermore the tar and virus cancers exhibit
precisely the same forms, some being "papillomas of the second order," some cystic,
while others are frankly malignant papillomas, and yet others are more or less
anaplastic squamous cell carcinomas (21).   A growth may assume all these
forms successively, or a form primarily or secondarily assumed may be long re-
tained, perhaps until death of the animal.   The metastases of both the tar and
virus tumors may be cystic[3] or solid, exhibit papillomatous features (Fig. 20) (4),
or be anaplastic.

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[2] Tar readily evokes squamous cell carcinomas in cottontail rabbits, as we
have found, and some of the cancers do not derive from papillomas but have the
carcinomatous form from the beginning.   Yet the possibility cannot be excluded
that they originated from carcinoids, themselves a manifestation of the spurious
malignancy of papillomas.   For carcinoids are frequently elicited in cottontails.

[3] The statement of a previous paper (*J. Exp. Med.*, 1936, 64, 401) that the
metastases of tar cancers are never cystic or papillomatous was based on informa-
tion since found to be incomplete, and it is negated by the instance of Fig. 20.

The tar cancers derive mostly from those papillomas which are broad-based (4), that is to say from those which are most vigorous; and it is from the most actively growing of virus papillomas that cancers arise oftenest. Continued stimulation of tar papillomas by further tarring hastens the occurrence of malignancy; and it is hastened in virus papillomas by a variety of stimulating procedures (21). Yet these are not essential in either instance. The vigor of virus papillomas as a rule greatly exceeds that of those due to tar; and, as might be expected, they become malignant much more often, and generally after a much shorter period, while the carcinomatous change is frequently multiple and cancers sometimes appear almost simultaneously at many spots in a single growth (21). Only an occasional tar papilloma ever becomes malignant.

Tarring so stimulates neoplastic proliferation in general that it may conceivably bring into the open some cancers which would not otherwise assert themselves, and which cannot progress after it is stopped. Yamagiwa and Ichikawa elicited a tar tumor with the morphology of a carcinoma, which metastasized yet retrogressed after 630 days (4); and amongst the numerous tar tumors of cottontail rabbits that we have recently evoked squamous cell carcinomas requiring aid have been frequent, growths which enlarged so long as tarring was kept up, not infrequently destroying a great part of the ear, yet which dwindled, though retaining the morphology of cancers, after it was left off, and eventually disappeared. In domestic rabbits growths of this sort are evidently rare, though the case just cited shows that they occur. Two large ulcerating cancers which had derived "spontaneously" from virus papillomas in our domestic rabbits, and were squamous cell carcinomas on biopsy underwent secondary, retrogressive changes. One had existed for months as a large, fungoid, malignant papilloma, distinctively different from the rest of the papillomatous mass amidst which it originated. Eventually it grew smaller and was almost replaced by the surrounding papillomatous tissue. The other cancer, an anaplastic, metastasizing squamous cell carcinoma, was at one time a bulky, ulcerated growth, yet dwindled in the end to a mere puckered induration over which the skin had healed. The microscope disclosed, however, that nests of living carcinoma cells still existed amidst a sclerotic connective tissue.

DISCUSSION

The benign tar tumors here described and classified were sharp cut pathological entities. They were not local exaggerations of the epidermal hyperplasia due to tarring, nor were they random neoplastic manifestations. One did not find papillomas grading into frill horns or *vice versa*. Whatever the nature of the cause for the frill horns, it expressed itself with an exquisite particularity, making the cells do precisely thus and so, with result in highly distinctive growths. The cause for the tar papillomas, though also acting upon the cells of the

stratum germinativum, produced tumors of wholly different sort, which usually expressed themselves in a characteristic, benign form, yet were often so responsive to external conditions as to assume instead a carcinomatous aspect without undergoing real cancerous change.

A comprehensive mapping of all the neoplastic potentialities of several animal species would greatly aid thought on tumor causation. But even in man only those potentialities are known today which have become actualities as result of the "carcinogenic" accidents of life. Observation has been largely haphazard in the case of other creatures, yet it already points to remarkable species differences. Tarring the skin of dogs, for example, results in malignant melanomas, apparently with some regularity (22), growths seldom if ever evoked in the rabbit or mouse. Tarring the skin of cottontails never results in frill horns, though frequently evoking papillomas, carcinoids, and cancers, as our extensive observations have shown. Can it be that the epidermis of different species of animals has different inherent potentialities for tumor formation? Or are these potentialities not inherent but due to agents of extraneous origin? Such questions can only slowly be answered.

The study here presented was made with hybrid rabbits of one sort (agouti) and with a single tar; but recently it has been extended to another breed (Dutch show rabbits). In these tar also evoked frill horns, papillomas, and carcinoids, with no other benign tumors, and in approximately the same relative number as in agouti rabbits. Furthermore a different tar has been applied to agouti rabbits and again the same tumors have been called forth, this time in an epidermis stimulated to much greater general change. The finding might have been expected in view of the evidence that tars owe their carcinogenic action to a common constituent, 1:2 benzpyrene. The pictures in the literature show that the domestic rabbits of England, America, Italy, Scandinavia, and Japan all yield on tarring papillomas and carcinoids resembling in general those with which we have dealt. But the differing fixatives and stains used on the specimens prevent any decision on whether the papillomas had the morphology of those of our animals. Some appear unlike them in details. Orr (23) reports that the growths evoked in mice by six carcinogenic hydrocarbons resemble those due to tar. It would be worth while to know the neo-

plastic effects of a single carcinogen upon rabbits of identical sort, bred in widely separate parts of the world, living under different conditions and fed different foods. (Our rabbits, all procured in New York, were housed and fed alike.) But in any typing of the growths thus evoked it would be necessary to discriminate their essential features from those due to intercurrent influences. This has not been done in the past, but instead all of the features of the tumors have been taken to be the expression of innate, individual peculiarities, and in consequence they have been deemed much more various than they actually are.

Because of the collateral influences that tar exerts it is far more effective in producing tumors in rabbits than any of the pure carcinogenic substances (10). It evokes growths much sooner than does benzpyrene even when this is applied in relatively large quantity, and it brings about local tissue changes which enable the tumors to become established. Directly or indirectly it urges them on and produces secondary changes in them. Because of this last influence tar tumors are best studied when young. Had we not examined them then, the narrow restrictions in their types might have been overlooked and the reasons for their later complexities have been missed.

The main facts emerging from the comparison of the benign tar and virus tumors are set forth in Table II, together with certain inferences which seem unavoidable. Our study was carried out on domestic rabbits, instead of cottontails, the natural hosts of the virus, to exclude all possibility that some of the growths evoked by tar might be due to a strain of virus lying latent and of such slight pathogenicity as to require the aid which tarring gives. For it is known that the papilloma virus may lie latent after introduction into normal skin and give rise to papillomas when tarring is done (10). The virus is wholly foreign to domestic rabbits and it cannot ordinarily be got again from the growths engendered in this species. Nevertheless the tarred rabbits were isolated to rule out contact infection entirely.

Serological tests indicate that the cause for the tar papillomas is not antigenically related to the Shope virus (24), and the distinctive cytological changes that it produces accord with this finding. To account for the general likeness to the virus tumors, one might

assume that anything which stimulates proliferation of the cells of the stratum germinativum will give rise automatically to papillomatous growth, if the proliferation is superficial. One may recall in this relation the likeness of silicosis tubercles to those due to bacillus

TABLE II*a*

Comparison of the Benign Tar and Virus Tumors Deriving from the Epidermis of Domestic Rabbits

The Tar Tumors	*The Virus Tumors*
Tarring evokes the growths	Tarring results in a localization of circulating virus: it enables latent virus to produce growths
Peculiar, chronic, "carcinogenic" tissue disturbances bring the unknown causes into action	Various non-specific, acute or chronic tissue disturbances render the virus effective
The growths appear on the tarred skin at irregular intervals	After a single intravenous injection of virus the growths all appear within a few weeks unless the skin has been often tarred, when they may appear irregularly

Number and time of appearance vary from host to host, but incidence is similar on both ears

Origin punctate or focal, but often multicentric

Enlargement takes place by intrinsic cell proliferation (*aus sich heraus*)

The tumors are largely dependent for persistence and growth on further tarring or on chronic changes already induced in the supporting tissues	The growths can progress without help of tarring or of chronic tissue changes, though aided thereby
Individual rates of proliferation vary widely; implantation elsewhere in the host is unsuccessful	Proliferation usually very rapid; implantation successful
Unfavorable local conditions frequently bring about general retrogression	Local conditions seldom cause general retrogression
Growths of two distinct types,—(1) papillomas subject to spurious malignancy = carcinoids, (2) frill horns	Growths are of a single sharply defined type,—papillomas

Inclusion bodies absent: no distinctive changes in the supporting tissue

Morphology indicates that they are due to specific causes, each with characteristic effects

Causes have not been recovered from the growths	Usually the virus cannot be recovered from the growths induced with it
The unknown causes require special cell conditions for their action, and exert little compulsion	*The virus cause is very compelling and does not require special aid*

TABLE II*b*

The Tar Papillomas	*The Virus Papillomas*
Both are sharply defined pathological entities	
Arise mostly from the deeper portion of the hair follicles	Arise mostly from the deeper portion of the hair follicles when the virus localizes out of the blood stream
Arise from the stratum germinativum	
Origin frequently multicentric	
Tumors enlarge in same way, assume same gross forms; virus papillomas usually more fleshy because growth more rapid	
Superficial resemblance close but cytology distinctive	
Occasional melanosis, and melanoblasts mildly stimulated. Pigmentation lost during rapid growth	Melanosis frequent, and melanoblasts greatly stimulated. Pigmentation often retained during rapid growth
Melanosis is of similar character	
Histological phenomena similar during retrogression	
Peculiarities of individual host influence gross form in same ways	
Aspect and course markedly influenced by local conditions	Aspect and course moderately influenced by local conditions
Continued tarring renders growths aggressive, complicates morphology	
Growths highly responsive to tarring. Spurious malignancy (formation of carcinoids) is a frequent result	Growths less responsive to tarring. It fails to induce spurious malignancy but other influences do this frequently
Retrogression usual unless chronic changes have been induced in the supporting tissues	Retrogression not infrequent; chronic tissue changes aid persistence
General retrogression frequent owing to local conditions; whether an induced resistance ever brings it about is uncertain	Local conditions have relatively slight effect, but general retrogression is frequent as result of induced resistance
Growths may reappear after vanishing	
Pathogenicity of cause, as expressed in tumor behavior, varies much from growth to growth	Pathogenicity of virus, as expressed in the multiple growths of a single infection, varies little
Occasional carcinomatous change after many months	Carcinomatous change frequent after a few months
Intercurrent stimulation hastens the malignant change	
The cancers arise from the papilloma cells by similar morphological alterations and exhibit the same general characters	
The cause of the papillomas stimulates the cells mildly and exerts a formative influence upon them which is often overborne. It has a moderate carcinogenic effect	*The virus stimulates the papillomas greatly and exerts a strict formative influence upon them. It has a pronounced carcinogenic effect*
The virus seems not to be related antigenically to the tar tumor cause yet the differences in the neoplastic phenomena they induce are merely quantitative	

tuberculosis. But tar and virus papillomas exhibit their characters though growing beneath the skin (Figs. 29, 30, 36, 37), while furthermore the frill horns, though submitted to similar mechanical influences and deriving from the stratum germinativum of hair follicles (Fig. 31), like the growths just mentioned, have a wholly dissimilar morphology (Figs. 8, 9).

Some of the tabulated differences in the tar papillomas and those due to virus are obviously consequent upon the great pathogenic activity of the latter, to its introduction into the test rabbits upon a single occasion, and to its almost standardized effects under ordinary circumstances. There is every reason to suppose that if we had mixed several virus materials of differing pathogenicity and injected the mixture several times, dribbling it into the animal, so to speak, the individual differences in incidence and behavior of the tar papillomas would have been duplicated.

The virus is a much more exigent formative influence than the unknown cause of the tar papillomas, the growths it produces retaining their morphology under conditions which would cause many tar papillomas to become carcinoids; but this difference would appear to be merely quantitative, since virus papillomas also become carcinoids on special occasion. In both instances the apparent malignancy means only that epithelial cells subjected to extraneous stimulation can simulate malignant cells, a fact already proven for normal epithelium by the invasive downgrowth with anaplasia which occurs after intradermal injection of Scharlach R or Sudan III in olive oil.

Though spurious malignancy is much more frequent in tar papillomas than in those due to virus, they are far less likely to undergo real malignant change, and the change generally takes place much later. The frequency with which cancer develops out of virus growths varies directly with how hard their cells are driven by the virus, as manifested by their rate of enlargement (25). The more rapid the proliferation, and the more the cells are played upon by extraneous stimuli (c.g. incision, Scharlach R injection, inflammation due to bacterial infection or vaccinial necrosis), the sooner and oftener does malignancy occur. The unknown cause of the tar papillomas exerts only a mild and conditional compulsion, as demonstrated by the behavior of the growths. This being so, one might expect that cancer would

arise late and infrequently from tar papillomas, as is generally the case. Yet to all appearance malignancy is the outcome of precisely the same train of events as in virus papillomas that are becoming malignant; and the resulting carcinomas are of the same kind and exhibit the same limited diversity. In a previous paper (21) we have discussed certain human cancers which arise from papillomas by changes resembling those which occur in rabbit papillomas, and have cited furthermore a virus of man which causes papillomatous proliferation out of which squamous celled carcinomatosis occasionally arises, namely the virus responsible for condyloma acuminatum (26). Like the unknown cause of the tar papillomas this virus fails to produce growths unless aided. Uncleanliness, local bacterial infection, tissue maceration, act to render it effective and to maintain the resulting condylomas, just as tar acts to evoke and maintain the tar tumors; and the cancers arise, as in their case, out of growths subjected to long-continued disturbance (27).[4]

SUMMARY

Tarring the ears of rabbits of one sort with a single kind of tar evoked epidermal tumors of a few sharply defined types, namely ordinary papillomas, carcinoids, carcinomas, and "frill horns." These last, relatively infrequent, are now recognized for the first time. The carcinoids have proved to be the expression of a spurious malignancy of papillomas, resulting from intercurrent influences, and they were wholly dependent upon these for their threatening aspect and behavior. Chief amongst such influences was continued tarring. It had the effect of establishing the papillomas, stimulated their proliferation, complicated their morphology, and rendered some of them disorderly, aggressive, and anaplastic. It brought all of the tissues of the ears into an excitable state, and often this state endured long after the skin had apparently returned to normal.

The characters of the papilloma-carcinoids and of the frill horns were so different and distinctive as to imply the action of differing, specific causes.

[4] The treatment of condyloma acuminatum has improved so greatly of late that secondary carcinomatosis has become exceedingly rare; but the conditions of its origin and development are amply documented by the numerous, well illustrated papers on the theme (27).

The papillomas were very like those induced with the Shope virus, and hence a point-to-point comparison was made of their manifestations, including the derivation of carcinomas from them. This comparison demonstrated that the unknown cause of the tar papillomas provoked neoplastic phenomena which were identical in all essential respects with those due to the virus.

To suppose, for experimental purposes, that the papillomas which tarring elicits are caused by a virus rendered pathogenic by this procedure, is to demand least of the unknown. Yet it does not follow that they must be due to a virus.

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EXPLANATION OF PLATES

All of the sections were stained with methylene blue and eosin.
V, T = virus and tar papillomas, respectively.

PLATE 20

FIG. 1. Tar papillomas. The inside of the ear had been tarred throughout several periods of some weeks each, with intervals between for recovery. Photographed on the 591st day, long after the last tarring. The skin between the growths is still somewhat scurfy in places. The tumors are far less fleshy than if tarring had been kept up, and hence do not resemble so closely the virus growths of Figs. 2 and 3. All those along the edge of the ear are papillomas, some medium gray (G), two almost black (G'). Several freckle-like patches of sooty, intracutaneous melanosis can also be seen. Near the middle of the ear is a small, recurved frill horn (H), and at one spot close to its edge is an aggregate of small subepidermal cysts (CY) resulting from the retrogression of a carcinoid. × ⅓.

FIGS. 2 and 3. For comparison with Fig. 1. Growths produced by directly infiltrating normal ears with papilloma virus by way of a marginal vein, and tarring them three times later, beginning after a week, in order to render the virus effective. (The uninfiltrated ears were also tarred: no growths ever appeared on them.) The papillomas of Fig. 2 were dry, cindery, and nearly all dark gray and slow growing, whereas those of Fig. 3, resulting from the same inoculum, were vigorous and fleshy, and about half of them were pigmented (G, G'). The dry tops of some of the others appear dark. Host influences have a great effect on the form of the growths. × ½.

The marginal situation of nearly all of the papillomas of Figs. 1, 2, and 3 is unusual. The specimens were selected for ease in photography.

FIG. 4. The melanotic virus papilloma (G') of Fig. 3,—for comparison with the melanotic tar papilloma of Fig. 5. The edge of the ear happens not to be incorporated in the growth. × 6½.

FIG. 5. Cross section of the melanotic tar papilloma, G' arrow, of Fig. 1. Its outer keratinized portion has been cut away. It extends around the edge of the ear on both aspects, with result that this is incorporated as a spurious raphe. × 6½.

FIGS. 6 and 7. Sections of a virus papilloma and a tar papilloma, respectively (V, T). Most of their dry keratinized portions have been trimmed away. The virus had been directly inoculated into the untarred skin of the side. × 13.

FIGS. 8 and 9. Frill horns, showing the characteristic narrow shape and transverse striation of the dry horns, and the fleshy collar about their bases. The pictures were taken long after tarring had been stopped. × 2½.

PLATE 21

FIGS. 10 and 11. Small frill horns. The sharply outlined, uninvasive basal frill of living epithelium and the compact, close-textured, keratinized material are alike typical. For convenience in sectioning, the dry horns have been broken off near the base. × 14 and × 10, respectively.

FIG. 12. Part of the base of a frill horn, to show the character of the cells, the absence of a stratum granulosum, and the abrupt keratinization with transient nuclear pycnosis. The scattered, irregular granules stippling the keratinized layer in some places, especially at the right of the photograph, have come from the breakdown of in-wandered polymorphonuclear leucocytes. More macrophages lie beneath the growth than is ordinarily the case. × 190.

FIG. 13. A typical anaplastic carcinoid, selected as illustrating the fact that such tumors are not necessarily preceded by papillomatous proliferation but may extend directly down from the surface epithelium. × 14.
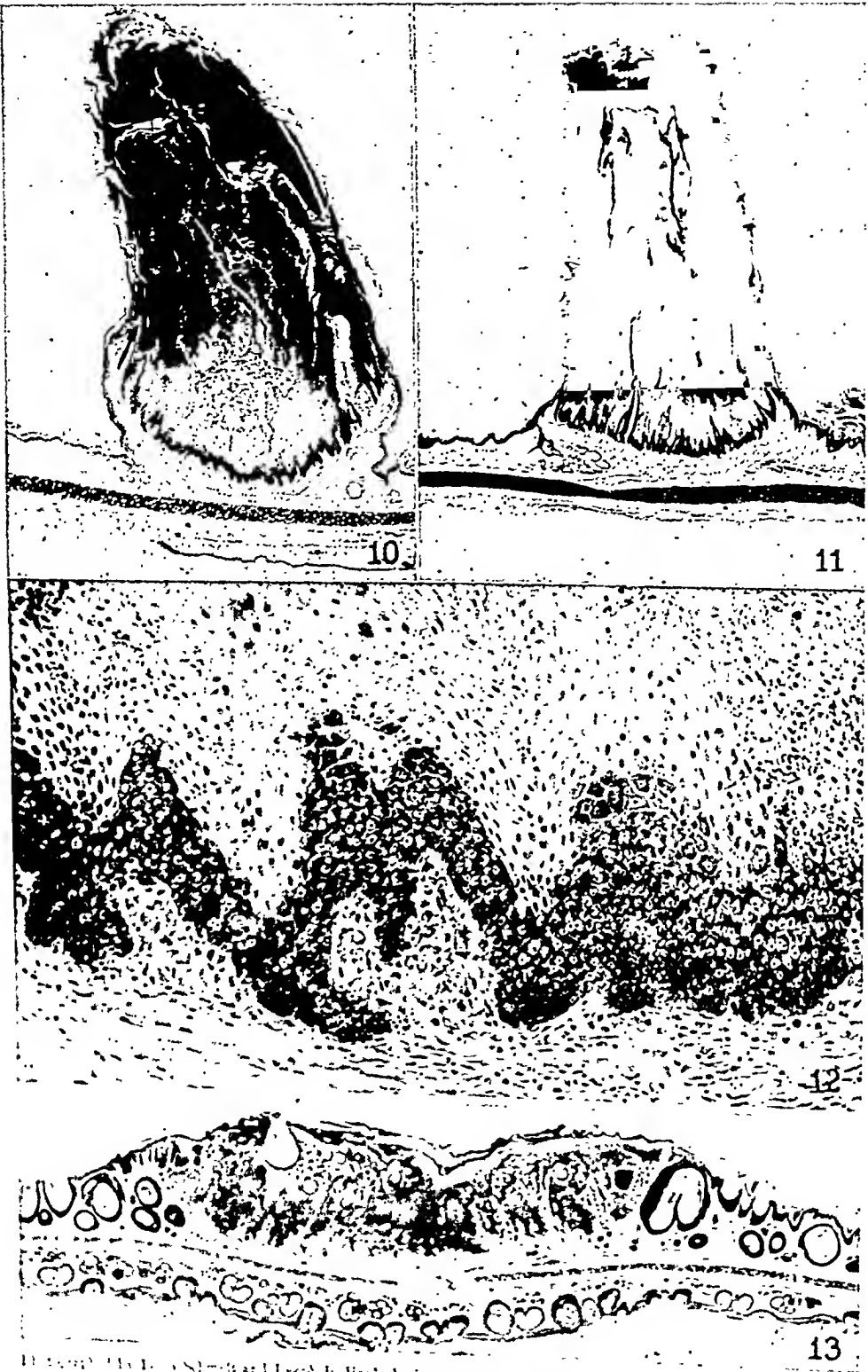
PLATE 21

Photo B. Hall. Comparison of class of local lesions with late tumor

PLATE 22

Fig. 14. A carcinoid of more organized type. × 18.

Fig. 15. Invasion of a large lymphatic by an anaplastic carcinoid which appeared after only one month of tarring and was then excised. The reactive connective tissue appears mucoid, as in the case of many squamous cell carcinomas. × 80.

Figs. 16 and 17. To illustrate the change of a carcinoid to a papilloma after the discontinuance of tarring. Fig. 16 shows a section across a biopsy specimen punched from a large carcinoid 10 days after tarring was stopped. The ear had dried down, and most of the growths, including the carcinoid, had begun to dwindle. It was already losing its anaplastic state, but deep in the reactive connective tissue beneath it were scattered, persisting groups of epithelial cells, not visible in the photograph. Fig. 17 shows the same tumor 28 days later, at the edge of the previous punch hole. Though it has extended around the edge to the outside of the ear, it is now an orderly keratinizing papilloma. The connective tissue beneath it has become sclerotic. × 21.

Fig. 18. Part of an early carcinoid. The rabbit had been tarred only 28 days yet the growth, an ulcerated dome, was already 8 mm. across. Half of it was punched out, as shown. It had invaded a large lymphatic (arrow). At this time there were 14 other growths on the ears, all subepidermal mounds or domes up to 9 mm. in diameter, and two of medium size, as yet unulcerated, were taken *in toto*. They showed carcinoids deriving from an intact epithelium. The ears were now stripped of tar for good. 12 days later only 4 growths remained, 3 as dry scabs, while the fourth, the other half of the carcinoid pictured, was reduced to a mere subepidermal thickening. It was punched out (see Fig. 19). After 2 weeks more all of the tumors had completely disappeared. × 14.

Fig. 19. The rest of the carcinoid of Fig. 18, as it appeared 12 days later. It now consists merely of keratinized cysts lined with stratified squamous epithelium devoid of any obvious neoplastic character. The end of the cartilage at the left marks the edge of the previous biopsy wound, but the scab over the healing tissue here has been torn away, together with a little of it. × 14.

Fig. 20. Section through the wall of a cystic metastasis from a tar carcinoma (see text). The growth was situated in an auricular lymph node. The living epithelium lining the cyst is papillomatous and keratinizing, but further away is anaplastic and notably invasive. × 44

PLATE 22

PLATE 23

FIGS. 21 and 22. Early stages of papillomatosis due to virus and tar, respectively,—to show that the growths began in the deeper portion of the hair follicles in both instances.

Fig. 21 is from an animal with ears prepared by tarring, which died 22 days after an intravenous virus injection. Massive infection with the virus took place, as shown by a sudden brawny swelling of the ears at about the 18th day (15), followed immediately by the appearance of growths. Autopsy disclosed that they were innumerable, some creamy but many dark. The section is from the outer side of the ear, which had been devoid of tar warts.

Fig. 22 is from an uninoculated animal which died after it had been tarred for 10 months, intermittently toward the end. At death it had numerous large, pedunculated papillomas on the tarred inner surface and a few small creamy-gray growths on the outside. Some that were just beginning at the latter situation are pictured. × 14.

FIGS. 23 and 24. Virus and tar papillomatosis at a slightly later stage. The resemblance is absolute at the magnification shown. A tar carcinoid (C) is included in Fig. 24. × 10.

To obtain Fig. 23, a normal rabbit ear was directly infiltrated with virus and tarred a few times later, as in the case of the ears furnishing Figs. 2 and 3. The specimen shown was punched from the ear 25 days after the infiltration, when innumerable pink or gray growths were appearing on both sides of the organ.

Fig. 24 came from the inside of an uninoculated ear tarred during a period of 80 days. Numerous growths had arisen, both papillomas and carcinoids.

FIGS. 25 and 26. A virus and a tar papilloma, situated on the outside of the ears, and just beginning to erupt. The virus papilloma was the result of a punctate inoculation into normal epidermis. The growth of Fig. 26 came from an animal tarred intermittently for 17 months. × 14.

(Ptaccord Eull. Compens and virus isolated tumors with tar tumors.)

PLATE 24

FIGS. 27 and 28. Further examples of erupting tar and virus papillomas. The growth of Fig. 27 was due to a punctate inoculation of virus. The tumor elicited by tar (Fig. 28) was from the same rabbit as Figs. 22 and 30. × 6½.

FIGS. 29 and 30. Somewhat larger virus and tar papillomas on the outside of the ear. The papillomatous pattern has become more complicated. The growth due to the virus resulted from direct inoculation, with tarring for 41 days thereafter. Fig. 30, of a tar papilloma, is from the rabbit, tarred for 10 months, that provided Figs. 22 and 28. × 14.

FIG. 31. Early stage of a frill horn; it is just erupting. The dense, strongly eosinophilic, keratinized material contrasts greatly with that formed by the virus and tar papillomas of Figs. 21 to 30, which stains almost not at all. The unstained whorls of keratin at the apex of the growth are the original contents of the distended hair follicle from which it arose. × 30.
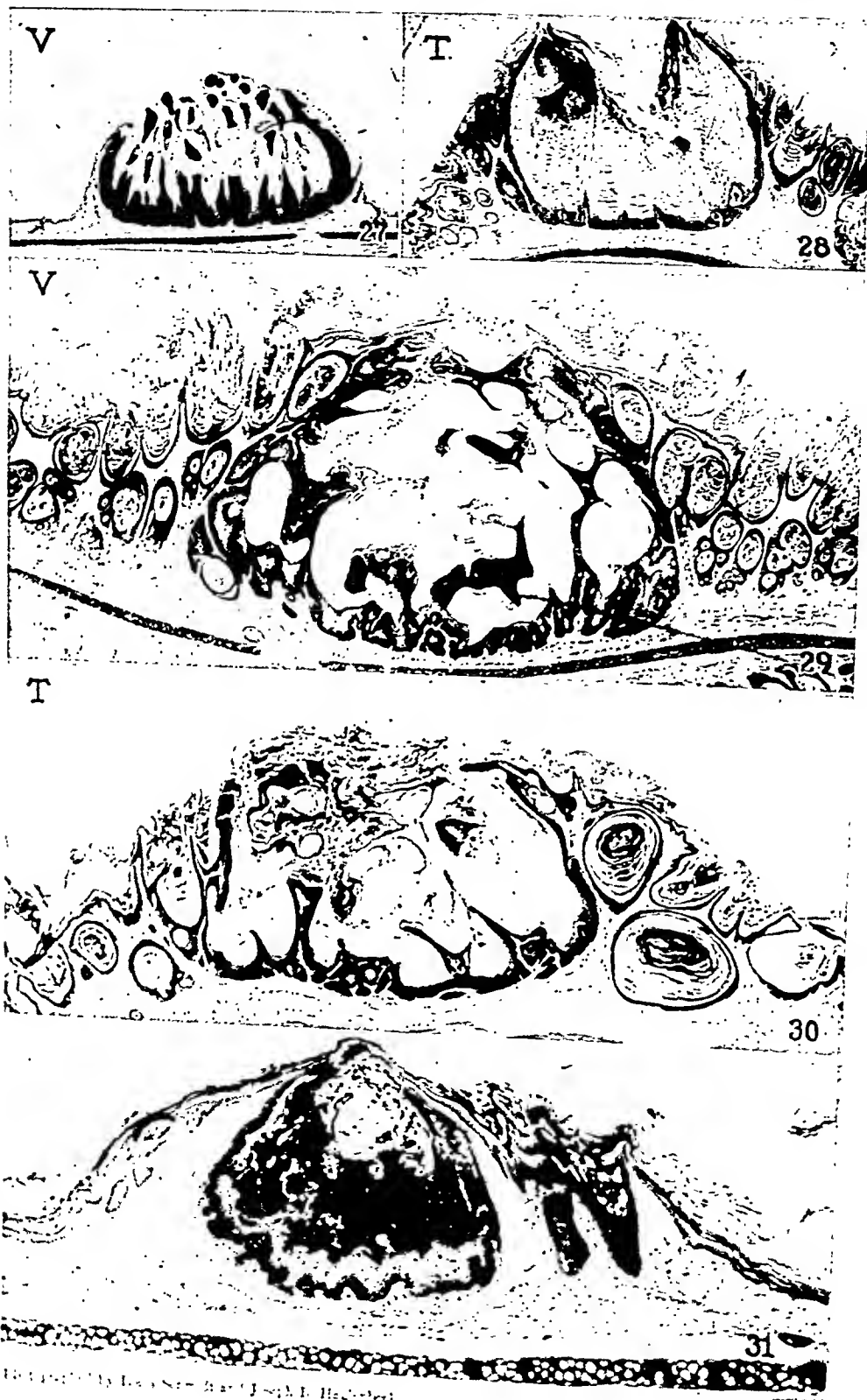
Figs. 32 and 33. The living epithelial layers of a non-pigmented virus and tar papilloma, respectively,—to show the likenesses and differences described in the text. × 177.

Figs. 34 and 35. The living layers of markedly melanotic virus and tar papillomas. The pigment-containing cells are morphologically alike in the two instances and occupy the same situations. × 168.

Figs. 36 and 37. Virus and tar papillomas which have extended through lacunae in the aural cartilage as result of the stimulus of continued tarring. The virus growth of Fig. 36 was the result of a tattoo inoculation into the inside of a normal ear 73 days previously, with tarring twice weekly thereafter. The growth became fungoid and foul, and extended through to the outside at several situations, of which two are seen here   Fig. 36, × 9.   Fig. 37, × 13.

Fig. 38. Extension of a tar papilloma to the outside of the ear. A punch biopsy disclosing the character of the growth was done early, and the hole that was left healed completely. The tumor became the large fleshy sphere that is pictured, and while doing so extended to the outside of the ear through the healed wound, with result in a fleshy "onion" there. Both growths consisted of connective tissue for the most part, covered with characteristic papillomatous epithelium, and with embedded islands of the latter.   × ½.

Figs. 39 and 40. Newly pedunculated virus and tar papillomas. The pedunculation was due to continued tarring. The virus tumor had been produced by a tattoo inoculation of a normal ear.   × 6.

Fig. 41. Final stage in the retrogression of a melanotic papilloma due to localization of circulating virus in an ear long tarred (15). The growth is almost gone but its place is marked by much intracellular pigment. The skin is everywhere pathological, its epithelium thickened, and the connective tissue unusually cellular. Scattered lymphocytes are present where the papilloma was once situated.   × 46.

PLATE 26

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ANTIGENIC PROPERTIES OF THE TYPE-SPECIFIC SUBSTANCE DERIVED FROM GROUP A HEMOLYTIC STREPTOCOCCI

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Although the constituent of the hemolytic streptococcus cell, which determines specificity in each of the various types included within group A, has been considered a protein, attempts to demonstrate its antigenicity after separation from the cell have not been entirely successful. This paper gives a more detailed study of this subject. It seems probable that in some instances the chemical procedures used to isolate the type-specific substances tended to degrade the proteins, and thus render them less antigenic. The early experiments (1) with the type-specific protein M showed that although the sera produced by the injection of whole streptococci gave specific precipitin reactions with this partially purified extract, the injection of the protein solution itself failed to stimulate type-specific antibody production in rabbits or guinea pigs, as shown by the following negative tests: precipitin reaction, agglutination, passive protection in mice, passive and active anaphylaxis in guinea pigs.

By extracting ground streptococci with increasingly alkaline solutions, Heidelberger and Kendall (2) isolated a fraction which stimulated type-specific precipitin formation when injected into rabbits; but these sera were not tested for their ability to protect mice passively.

Mudd and his collaborators (3) have reported the isolation of an antigenic fraction from group A hemolytic streptococci which they have named labile antigen.[1] They postulated that the labile antigen is a complex molecule containing the type-specific protein M in addition to several other serologically active constituents. Besides absorbing type-specific agglutinins, opsonins, and protec-

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[1] On the basis of recent unpublished findings of Mudd, Lackman, Pettit, and Morgan, Dr. Mudd has informed us that these authors now prefer to substitute the term "nucleoprotein agglutinogen" for "labile antigen."

tive antibodies from antibacterial serum, the labile antigen, when injected into rabbits, induced antibodies which gave partly type-specific precipitin reactions, but these antisera were not tested for protective action in animals.

In 1937, Stamp and Hendry (4) isolated a fraction from a group A type 3 hemolytic streptococcus (strain Richards), which produced active immunity when injected into mice. 47 per cent of the immunized animals were protected against 100 minimal lethal doses of the homologous organism. The specificity of this immune response, however, was not tested.

EXPERIMENTAL

As a starting point for obtaining the type-specific substance in antigenic form, it was thought promising to use bacteria ground in the cold, as suggested by Mudd and his collaborators. The first untreated saline extracts of such material, injected into rabbits, induced the formation of slight amounts of antibody, as demonstrated by precipitin and agglutinin tests and the passive protection of mice, but the antibody titers of these sera were very low.

Because of the large quantities of extract required to immunize rabbits, it was decided to immunize mice and to test their active immunity following the method of Stamp and Hendry, using the per cent of survival of actively immunized mice as the index of antigenicity of various preparations. In numerous experiments this technique proved to be a rough but convenient way of testing various fractions.

Methods

Selection of Strains.—Since the antigenicity of the extracts was to be tested by actively immunizing mice, it was essential to use only highly mouse-virulent strains which would be suitable for testing the degree of immunity in mice. It seemed probable, furthermore, that virulent cultures would yield larger amounts of antigenic material than avirulent ones. One strain each of three different types within group A was used for preparing antigenic extracts and one additional strain of each of these types was employed for testing the immunity.

Description of Strains.[2]—

Type 1:[3] 1. *Strain S118* was isolated in Texas in 1918 from the pleural fluid of a patient with bronchopneumonia following measles (6).

2. *Strain T1* is Griffith's type 1 strain, S. F. 130/2 (7).

Type 3: 1. *Strain D58* is the strain Richards, isolated by Colebrook from

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[2] Strains T1 and T14 were kindly sent by Dr. F. Griffith, strain D58 by Dr. T. C. Stamp, and strain C203 by Dr. M. B. Kirkbride.

[3] Types are designated according to Griffith's classification (5).

puerperal septicemia (4). It was obtained in a virulent state following mouse passage.

2. *Strain C203*[4] was isolated by Dochez about 1921 from a patient with scarlet fever, and obtained in virulent form in 1927.

Type 14: 1. *Strain S23* was isolated in Texas in 1918 from the throat of a patient with lobar pneumonia (6).

2. *Strain T14* is strain Barker, a representative of type 14.

Virulence.—At the beginning of this experiment, three of the six strains, namely S23, C203, and D58, were virulent enough to kill mice in doses of 10^{-6} cc. to 10^{-8} cc. of 6 to 12 hour cultures. Strain T14 was moderately virulent and required only six to eight mouse passages to reach the same degree of virulence. The other two strains, S118 and T1, were so degraded that they produced typically glossy colonies and failed to yield demonstrable type-specific substance in extracts of ordinary concentration. They were so avirulent that 0.1 cc. to 0.5 cc. of a young culture was required to kill mice. By repeated mouse passage (25 passages for S118 and 60 passages for T1) it was possible to render these cultures virulent.

The chief method used in the preparation of antigenic extracts was as follows:—

Medium.—The bacteria used for preparing antigenic extracts were grown in the type of broth developed by Todd and Hewitt (8), modified chiefly by the substitution of beef heart for horse meat. The fact that the broth is sterilized by filtering through Chamberland B filters, rather than by heating, increases its value as a medium but also adds an element of danger in its use, since contaminants sometimes grow in uninoculated flasks which have been incubated for 3 or 4 days. By seeding the broth immediately after filtration with a very large inoculum, and then limiting the incubation to 4 hours, pure cultures were obtained in all except one instance.

During the early stages of growth in this broth, large capsule-like areas could be seen surrounding the organisms in moist India ink preparations. These capsules increased markedly in size and were maximal in about 4 hours, following which they rapidly became smaller. The bacteria at this early stage were very difficult to throw down in the centrifuge, but following heating at 56°, or after more prolonged incubation, the capsules disappeared and the bacteria were then easily packed on centrifugation, an observation also made by Seastone (9) and more recently by Loewenthal (10).

These capsules resembled those described by Seastone and later by Ward and Lyons (11), in young cultures of hemolytic streptococci grown in whole blood, in that they were present only in young cultures and were not correlated with

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[4] While Dr. Griffith has placed this strain in type 1 by means of slide agglutination, work carried out in this laboratory indicates that, on the basis of protection and precipitin tests, it falls in type 3. Further studies are being made here on the interrelationships of strain C203 and types 1 and 3.

mouse virulence, but they differed in that we could not stain them by ordinary methods. The difficult question of the significance of capsules for the hemolytic streptococcus has been reviewed by a number of authors (9, 10, 12), but in these experiments the presence of a capsule was taken to mean that the cultures were in a period of optimal physiological activity.

Collection of Bacteria.—The broth was filtered while still hot through Chamberland filters and was then cooled to 37°C. 64 liters were inoculated with 25 cc. per liter of an actively growing 4 hour culture. After 4 hours' growth, samples were removed from each flask and plated on blood agar to test the purity of the culture. The culture was then stored at 0°C. overnight, and kept chilled while running through a Sharples centrifuge on the following day. The caked bacterial sediment was suspended in about 200 cc. of cold saline by grinding it in a large, previously chilled mortar and pressing it through sterile gauze, stretched over a wire strainer, into another cold container in order to break up clumps and facilitate uniform heating during the process of heat-killing. The suspension was then transferred to a glass stoppered pyrex bottle, and the bacteria killed quickly by the following procedure: A thermometer was inserted into the bottle so that the temperature could be accurately adjusted. The temperature was raised within 1 minute to 56°C. by the addition, while shaking, of about 400 cc. of boiling saline. The stopper was then covered tightly with a rubber cap; and the bottle was completely immersed for 15 minutes in a 56°C. water bath. Sterility tests showed that all the bacteria in this heavy suspension were killed by the end of this time. The suspension was rapidly cooled by placing the bottle under running water and then centrifuged in 50 cc. wide mouthed tubes; the supernatant fluid was discarded. The tubes, containing not more than 3 gm. dry weight of bacterial sediment, were placed in a CO_2-ice-acetone mixture. While freezing, the bacteria were easily distributed over the sides of the tube with a spatula. The completely frozen organisms were dried by means of the Flosdorf-Mudd lyophile apparatus (13).

Extraction.—Preparatory to extraction, the bacteria were ground in a ball mill consisting of a 1 liter spherical heavy glass flask and 500 one-quarter inch stainless steel balls (14). At first the grinding was done at $-73°C$. as advocated by Mudd (15). Later, however, it was found that satisfactory results could be obtained by grinding 1 gm. of dried organisms in each flask for one-half hour at room temperature. This procedure rendered approximately 75 per cent of the cocci Gram-negative without causing much change in their morphology. The organisms from twelve flasks were collected in 500 cc. N/10 HCl and extracted at 37°C. for 24 hours. After centrifugation the supernatant extract was removed; 250 cc. N/10 HCl were added to the organisms which were then extracted for a second 24 hours. This procedure was repeated on a 3rd day. The yield of active material from the second and third extractions tended to be larger than that from the first; but since further extractions resulted in smaller yields, the bacterial residue was discarded.

Purification of the Extract.—On cautious neutralization of each of these acid

extracts with N/1 NaOH a precipitate began to form as pH 4.0 was approached and became maximal at about pH 4.5. In the case of the first acid extract it was necessary to bring the pH to 5.0 before flocculation occurred. After standing overnight in the ice box the precipitates were thrown down in a centrifuge and the supernatant fluids discarded. The combined precipitates from various extractions were taken up in M/15 phosphate buffer solution at pH 7.2 but most of the precipitate was insoluble and was discarded. N/1 HCl was slowly added to the supernatant fluid. As pH 5.5 was approached a precipitate began to form which was maximal at pH 4.5. This was allowed to stand overnight in the ice box and the precipitate separated the following morning. The supernatant fluid was discarded, and now nearly all the precipitate was dissolved in 50 cc. of M/15 phosphate buffer at pH 7.2. After removal of the small amount of insoluble material the solution was filtered through a Berkefeld N filter.

The filtrate was distributed in amounts suitable for one day's injections and then frozen and dried to prevent deterioration. In order to estimate the dosage, the total nitrogen and that precipitated by trichloroacetic acid were determined by means of the micro Kjeldahl method. The nitrogen precipitated by trichloroacetic acid was always between 50 and 60 per cent of the total nitrogen. Since it was felt that the antigenic activity probably resided in the protein fraction, the dosage in all experiments was calculated as 6.25 times the nitrogen content of the material precipitated by trichloroacetic acid; and the total yield calculated in the same way was about 0.6 per cent of the dry weight of the bacteria.

Serological Reactions Obtained with the Extract.—Different preparations of antigen reacted type specifically to about the same dilution, usually 1:200,000 in precipitin tests with homologous type-specific sera. The dilution was calculated in the same way as the dosage employed in immunization. No group-specific polysaccharide C could be detected in these extracts when tested with sera potent in anti-C precipitins nor could this carbohydrate be split off by heating the solution at 100°C. with N/20 HCl.

The method of extraction and partial purification outlined was adopted as the procedure of choice for the chief experiments of this investigation, but several less successful methods of preparation were tried in numerous other experiments.

Active Immunization of Mice

The mice used in these experiments were the Rockefeller strain. When possible, mice weighing 24 to 26 gm. were used for active immunization, since they seemed to give better results than smaller ones. The dried antigenic extracts were dissolved in physiological sodium chloride solution just before injection, which was made intraperitoneally on 3 successive days with a rest period of 4 days between courses. Usually three or four courses were given. The immunized and untreated control mice were inoculated with the test culture 1 week

after the last injection of the antigen. Experiments were terminated after 2 weeks' observation.

The mice in the first experiment (Table I) were immunized for 4 weeks with an

TABLE I

Active Protection Test in Mice

Titer of Homologous Immunity

Mouse No.	Mice immunized with extract of type 1 strain, S118						Untreated controls	
	Inoculated with the following amounts of S118							
	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-7} cc.	10^{-8} cc.
1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2	D 1 "	D 1 "	D 2 days	D 1 "	D 3 days	D 3 days	D 1 "	D 1 "
3	D 1 "	D 1 "	D 2 "	D 1 "	D 3 "	D 5 "	D 1 "	D 1 "
4	D 1 "	D 1 "	D 4 "	D 2 days	D 5 "	S	D 1 "	D 1 "
5	D 1 "	D 1 "	D 6 "	D 2 "	D 6 "	S	D 1 "	D 1 "
6	D 1 "	D 1 "	D 7 "	D 2 "	S	S	D 1 "	D 1 "
7	D 1 "	D 1 "	D 10 "	D 3 "	S	S	D 1 "	D 1 "
8	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
9	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
10	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
11	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
12	D 1 "	D 3 days	S	S	S	S	D 1 "	D 1 "
13	D 1 "	D 4 "	S	S	S	S	D 2 days	D 1 "
14	D 1 "	S	S	S	S	S	D 2 "	D 2 days
15	D 1 "	S	S	S	S	S	D 3 "	D 3 "
16	D 1 "	S	S	S	S	S	D 3 "	D 3 "
17	D 1 "	S	S	S	S	S	S	D 3 "
18	D 1 "	S	S	S	S	S	S	D 3 "
19	D 1 "	S	S	S	S	S	S	D 5 "
20	D 1 "	S	S	S	S	S	S	S

The mice were immunized for 4 weeks: 3 injections of 0.01 mg. each were given the 1st week, 3 of 0.02 mg. each the 2nd, 3 of 0.04 mg. each the 3rd and 4th.

The protection tests were performed as follows: A fresh 16 hour broth culture was serially diluted with broth so that the amount inoculated was contained in 0.5 cc. The inoculations were intraperitoneal. The untreated controls corresponded in age and weight with the immunized animals. The number of streptococci injected was estimated in colony counts from poured blood agar plates containing 10^{-6} cc., 10^{-7} cc., and 10^{-8} cc., respectively, of the culture used for inoculating the mice. The number of colonies in 10^{-8} cc. varied from two to six in different experiments.

In all experiments S indicates animals which survived at least 2 weeks, and D indicates death on the day stated.

extract of strain S118 in the manner indicated in Table I.   A total dosage of 0.33 mg. was given to each animal.   1 week after the last injection, they were divided into six groups of 20 mice each, and each group was injected with a different dose of an overnight culture of strain S118.   None of the mice receiving 1,000,000 M.L.D. (10^{-2} cc.) survived, but in all the groups receiving smaller doses enough animals survived to show definite protection against the homologous organism.

The second experiment (Table II) was designed to test whether the active immunity induced by these antigens was type-specific.   Three sets of 84 mice were immunized for four courses with extracts of streptococci of three different types (types 1, 3, and 14).   The mice received the same amounts of antigenic extract as those in the first experiment.   1 week following the last dose of antigen each group was subdivided into six subgroups of 14 mice each.   Six strains were used as test inocula, two each of types 1, 3, and 14.   Each subgroup received 10^{-6} cc. of culture diluted in broth.   This small dose, containing 10 to 100 M. L. D., which killed all the control animals regularly, was selected in order to detect even slight evidence of cross protection.

In each case, good protection was demonstrated against 10 to 100 lethal doses of streptococci of the homologous type, as there were 93 per cent survivors in type 1, 93 to 100 per cent in type 3, and 43 to 50 per cent in type 14.   On the other hand, comparably immunized mice, when inoculated with heterologous strains, showed only slight or no immunity.   Some protection against the type 1 cultures was afforded to mice immunized with heterologous extracts, but little if any cross immunity was found where types 3 and 14 were used as test inocula.   In every case type-specific immunity was clearly greater than the immunity against heterologous types.   It is probable that if a larger dose of culture had been used, the immunity induced by immunization with extracts would have appeared strictly type-specific.

Type-Specific Immunization of Rabbits with Extract

After completing the experiments on active immunity in mice, an attempt was made to immunize rabbits with the same antigenic extract.   The immunity was tested by protection of mice by the sera of these animals.   The rabbits were treated as shown in Table III.   During immunization the response to the antigen was determined by precipitin tests with the homologous M extract.

These tests showed that the sera of the two rabbits immunized with S118 extract (rabbits R47-08 and R47-09) contained no precipitins against the homologous extract at the end of the third course, but both had precipitins and protective antibodies at the end of the fifth course.   The rabbit given seven courses showed no increase in antibody titer over that reached at the end of the fifth series.   The sera of the two rabbits (R47-22 and R47-23) similarly immunized with an

TABLE II

Active Protection Test in Mice

Type Specificity of Immunity

		Immunized mice					
		Inoculated with 10^{-6} cc. of					
Immunized with extract of strain	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
S118 (type 1)		(Homologous)					
	1	D 7 days	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	3	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	4	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	5	S	S	D 1 "	D 1 "	D 1 "	D 2 days
	6	S	S	D 1 "	D 1 "	D 2 days	D 2 "
	7	S	S	D 1 "	D 1 "	D 2 "	D 2 "
	8	S	S	D 2 days	D 1 "	D 3 "	D 2 "
	9	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	10	S	S	D 2 "	D 2 days	D 3 "	D 3 "
	11	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	12	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	13	S	S	D 3 "	D 2 "	D 9 "	D 3 "
	14	S	S	D 3 "	D 2 "	S	D 5 "
D58 (type 3)				(Homologous)			
	1	D 1 day	D 1 day	S	D 3 days	D 1 day	D 1 day
	2	D 1 "	D 1 "	S	S	D 1 "	D 2 days
	3	D 1 "	D 1 "	S	S	D 1 "	D 2 "
	4	D 2 days	D 2 days	S	S	D 1 "	D 2 "
	5	D 2 "	D 2 "	S	S	D 2 days	D 2 "
	6	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	7	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	8	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	9	D 3 "	S	S	S	D 3 "	D 2 "
	10	D 3 "	S	S	S	D 3 "	D 2 "
	11	S	S	S	S	D 3 "	D 3 "
	12	S	S	S	S	D 7 "	D 3 "
	13	S	S	S	S	D 7 "	D 3 "
	14	S	S	S	S	S	D 8 "
S23 (type 14)						(Homologous)	
	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
	3	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
	4	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 2 "
	5	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 3 "
	6	D 2 days	D 2 days	D 1 "	D 1 "	D 6 "	D 3 "
	7	D 2 "	D 2 "	D 1 "	D 1 "	D 7 "	D 3 "
	8	D 2 "	D 2 "	D 1 "	D 1 "	S	D 9 "
	9	D 2 "	D 2 "	D 1 "	D 1 "	S	S
	10	D 2 "	D 2 "	D 1 "	D 2 days	S	S
	11	D 2 "	D 3 "	D 1 "	D 2 "	S	S
	12	S	D 3 "	D 1 "	D 2 "	S	S
	13	S	S	D 2 days	D 2 "	S	S
	14	S	S	D 2 "	D 2 "	S	S

TABLE II—*Concluded*

Mouse No.	Dose	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
	cc.						
1	10^{-6}	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
4		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
5		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
6		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
7		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
8		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
9		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
10		D 1 "	D 1 "	D 1 "	D 2 days	D 2 days	D 2 "
11		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
12		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
13		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
14		D 5 days	D 1 "	D 2 days	D 15 "	D 2 "	D 7 "
1	10^{-7}	D 1 day	D 1 "	D 1 day	D 1 day	D 1 day	D 2 "
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
4		D 1 "	D 1 "	D 1 "	D 2 days	D 2 "	D 2 "
5		S	D 1 "	D 3 days	D 9 "	S	D 2 "
1	10^{-8}	D 1 day	D 2 days	D 1 day	D 2 "	D 2 days	D 1 day
2		S	D 2 "	D 1 "	D 2 "	S	D 2 days
3		S	D 2 "	D 2 days	D 2 "	S	D 2 "
4		S	S	D 2 "	D 2 "	S	D 2 "
5		S	S	S	S	S	S

extract of strain D58, contained demonstrable type-specific precipitins and protective antibodies after 3 weeks' immunization; and the titer was increased following 2 weeks' further immunization.

The capacity of these sera to protect mice was tested with six different strains of hemolytic streptococci, representing three types (Table IV). The S118 serum was a pool of bleedings from rabbit R47-08 after the fifth and seventh courses of injections of antigen. The D58 serum was taken from rabbit R47-23 after the fifth course. Although the cultures used in the passive protection experiments were

somewhat less virulent than usual, as shown by the survival of some control mice, nevertheless, there was distinct protection against 1,000 to 100,000 m.l.d. of the homologous type streptococci with some irregular deaths. The few survivals among animals tested with heterologous strains were very irregular, and the results in general show strict type specificity in these passive protection tests.

TABLE III

Protocol of Immunization of Rabbits with Extracts

Course of injection	Rabbits R47-08 and R47-09* with S118 extract	Rabbits R47-22 and R47-23 with D58 extract
1st	2.5 mg. daily for 5 days	2.5 mg. daily for 5 days
2nd	4.3 " " " " "	2.5 " " " " "
3rd	5.0 " " " " "	5.0 " " " " "
	Test bleeding	Test bleeding
4th	10.0 mg. daily for 5 days	5.0 mg. daily for 5 days
5th	10.0 " " " " "	10.0 " " " " "
	50 cc. bleeding	Final bleeding
6th	10.0 mg. daily for 5 days	
7th	20.0 " " " " "	
	Final bleeding	

Immunizing material was dissolved in saline, 1 to 2 cc. and given intravenously. There were 2 days of rest between each course except where a bleeding was taken, in which case the interval was a week.

* The final bleeding from rabbit R47-09 was taken after the sixth course of injections.

Precipitin Reactions with Anti-Extract Sera

In Table V are shown the precipitin reactions with samples of the same sera used in the passive protection tests recorded in Table IV. The M substances used as reagents were extracted with N/20 HCl in a boiling water bath as previously described (16). The immediate reactions were strikingly type-specific, but on standing overnight in the ice box the somewhat confusing cross reactions appeared. The latter are recorded in the table. The type 1 serum was not very potent but reacted most strongly with extracts of the homologous type strains, S118 and T1. It also gave weak reactions with all three of the type 3 extracts used but none with the type 14 extracts. The much more potent type 3 serum gave good immediate precipitates

with extracts of all the homologous type strains (T3, D58, C203), and weaker reactions with the type 1 extracts. With type 14 extracts, the type reactivity of which had been previously established by testing with antibacterial sera, this serum (R47-23) gave only traces of precipitin reaction. Neither serum contained group-specific antibody, as indicated by their failure to precipitate with a solution of group-specific C polysaccharide which, in the dilutions used, regularly precipitated sera known to contain the group-specific anti-C precipitin.

It is highly probable that the cross reactions in the precipitin tests are due to the presence of non-type-specific antibodies in the rabbit serum and of non-type-specific precipitinogens in the M extracts used.

Absorption of Antibacterial Serum with Extracts

In order to test the evidence identifying this antigen with the substance in intact streptococci which stimulates the production of protective antibodies in rabbits, the antigen was used to absorb protective antibody from a serum made by immunizing a rabbit with whole streptococci. Since Mudd and his collaborators (3) state that the type-specific protective antibody cannot be absorbed by the M fraction, extracted with N/20 HCl in a boiling water bath, another sample of the serum was absorbed with the M substance so prepared.

A rabbit was immunized chiefly with heat-killed but also with living culture of the type 1 strain, T1. Its serum gave a strong precipitin reaction with the homologous M antigen, and regularly protected mice against 1,000,000 M.L.D. of type 1 strains. One portion of serum was absorbed with a known antigenic extract of strain S118 (lot 61, made in the same manner as that used as antigen for the active and passive immunization experiments). Another portion was absorbed with an M extract of strain S118 made with N/20 HCl in a boiling water bath. Neither solution contained demonstrable group-specific C polysaccharide. The optimal proportions point for the precipitation of the serum by each antigen was determined by titration (17), and the solutions of antigens were added to the respective sera in double the optimal proportions. After incubating the mixtures at 37° for 2 hours and keeping them in the ice box overnight, the precipitates were removed and discarded and more of the respective antigen was added to the partially absorbed sera. Practically complete absorption was indicated by the lack of further precipitation with the lot 61 antigen and by the mere trace of precipitate with the M extract prepared by the older method. Serial dilutions of each absorbed serum and of a control lot of the same serum unabsorbed were

TABLE IV

Passive Protection Test in Mice—Type Specificity of Immunity

Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
cc.							
		(Homologous)					
10^{-3}	1	D 1 day	D 1 day				
	2	D 1 "	D 1 "				
	3	S	S				
	4	S	S				
10^{-4}	1	D 3 days	S				
	2	S	S				
	3	S	S				
	4	S	S				
10^{-5}	1	D 3 days	S	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 4 "	S	D 1 "	D 1 "	D 2 days	D 1 "
	3	S	S	D 1 "	D 1 "	D 4 "	D 8 days
	4	S	S	D 2 days	D 1 "	D 4 "	S
10^{-6}	1	D 2 days	S	D 1 day	D 1 "	D 3 "	D 2 days
	2	S	S	D 1 "	D 1 "	D 3 "	D 4 "
	3	S	S	D 2 days	D 1 "	D 9 "	D 5 "
	4	S	S	D 2 "	D 2 days	S	S
10^{-7}	1	S	S	D 2 "	D 1 day	D 2 days	D 3 days
	2	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	3	S	S	D 3 "	D 2 days	D 4 "	D 6 "
	4	S	S	D 3 "	D 2 "	S	S
Virulence controls: no serum							
10^{-5}	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	S
	3	D 1 "	D 1 "	D 2 days	D 1 "	D 4 days	S
	4	D 1 "	S	D 2 "	D 1 "	S	S
10^{-6}	1	D 1 "	D 1 day	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	D 1 "	D 2 days	D 1 "	D 1 "	D 1 "
	3	D 2 "	D 1 "	D 2 "	D 1 "	D 2 days	D 2 days
	4	D 2 "	S	D 2 "	D 1 "	S	S
10^{-7}	1	D 1 day	D 3 days	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	S	D 2 days	D 1 "	D 1 "	D 2 days
	3	D 2 "	S	D 2 "	D 1 "	S	S
	4	S	S	D 2 "	D 10 days	S	S
10^{-8}	1	D 3 days	D 1 day	D 5 "	S	S	S
	2	S	S	S	S	S	S
	3	S	S	S	S	S	S
	4	S	S	S	S	S	S

TABLE IV—*Concluded*

Serum from rabbit R47-23 immunized with D58 (type 3) extract

Mice inoculated with test culture

Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
cc.							
				(Homologous)			
10^{-1}	1			D 1 day	D 1 day		
	2			D 1 "	D 1 "		
	3				D 1 "		
	4				S		
10^{-2}	1			D 1 "	D 1 day		
	2			D 1 "	D 1 "		
	3			D 1 "	S		
	4			S	S		
10^{-2}	1			S	D 2 days		
	2			S	D 3 "		
	3			S	S		
	4			S	S		
10^{-4}	1			D 1 day	D 1 day		
	2			D 7 days	S		
	3			S	S		
	4			S	S		
10^{-c}	1	D 1 day	D 1 day	S	D 10 days	D 1 day	D 1 day
	2	D 2 days	D 1 "	S	S	D 1 "	D 1 "
	3	D 2 "	D 1 "	S	S	D 2 days	D 1 "
	4	D 2 "	D 1 "	S	S	D 2 "	D 1 "
10^{-c}	1	D 1 day	D 12 days	S	S	D 1 day	D 1 "
	2	D 2 days	S	S	S	D 1 "	D 1 "
	3	S	S	S	S	D 1 "	D 1 "
	4	S	S	S	S	S	D 2 days
10^{-7}	1	D 3 days	S	S	S	D 1 day	D 1 day
	2	D 3 "	S	S	S	D 1 "	D 2 days
	3	D 3 "	S	S	S	D 2 days	D 3 "
	4	S	S	S	S	S	D 5 "

18 to 20 gm. mice were injected intraperitoneally with 0.5 cc. of the serum indicated, the day before inoculation. Fresh 12 hour blood broth cultures were diluted serially with broth so that 0.5 cc. contained the desired dose. The amounts recorded were injected intraperitoneally into four mice in each set. Similar sets of mice which had received no serum were included as virulence controls.

given to mice. On the following day all the mice were inoculated with varying amounts of strain S118.

TABLE V

Precipitin Reactions

Antisera Prepared with Extracts

Serum		M extracts for precipitin tests							Group-specific C fraction
		Type 1 strains		Type 3 strains			Type 14 strains		
		S118	T1	D58	C203	T3*	S23	T14	
	cc.								
Rabbit R47-08 anti-S118 extract (type 1)									
0.2 cc.	0.4	—	++	±	±	+	—	—	—
"　"	0.2	—	++	+	+±	+	—	—	—
"　"	0.1	+	+	+	±	—	—	—	—
"　"	0.05	+±	+±	—	±	—	—	—	—
"　"	0.025	+±	++	—	—	—	—	—	—
"　"	0.013	+±	++	—	—	—	—	—	—
Rabbit R47-23 anti-D58 extract (type 3)									
0.2 cc.	0.4	++	++	+++	++	++	+	±	—
"　"	0.2	+±	+±	+++	+++	+++	+	+	—
"　"	0.1	+±	+±	+++	+++	+++	±	+	—
"　"	0.05	+	+	+	+±	++	±	—	—
"　"	0.025	±	±	±	+	+±	±	—	—
"　"	0.013	±	±	±	+	±	—	—	—

The M extracts used here were prepared by heating at 100°C. with N/20 HCl and after alcohol precipitation were made up in a final concentration of about one-fifth of the original volume of extracts. Serial dilutions were made with saline in 0.4 cc. volume. To each tube 0.2 cc. of serum was added, they were then incubated at 37° for 2 hours and kept in the refrigerator overnight before reading. Readings were made on a scale of ++++ to ±. The C fraction used here was made from the type 6 strain S43 by acid extraction and the protein removed with HgCl₂. In the concentrations used here it gave strong precipitin reactions with sera potent in anti-C.

Strain S43 was isolated in Texas in 1918 from the throat of a patient with measles.

* Strain T3 is Griffith's strain "Lewis opaque," kindly sent by Dr. Griffith as a representative of type 3. It was given 40 passages through mice to increase its virulence and its content of type-specific substance.

The results of the absorption experiment recorded in Table VI, indicate that the lot 61 extract removed the protective antibody from the antibacterial serum so that even in doses of 0.5 cc. the absorbed serum no longer protected against as little as 100 M.L.D. (10^{-5} cc.) of a strain of homologous type.   The absorption with the M substance, extracted at 100° with N/20 HCl, was only slightly less complete: 0.5 cc. of this absorbed serum protected only against 100 and 1,000 M.L.D. and not against larger doses of culture.   This amount of protection was insignificant when compared with the original titer of the serum which was high enough for 0.06 cc. to protect a mouse against 100,000 M.L.D.

Active Immunization of Mice with M Extracts

The hypothesis that the M substance is essentially similar to the antigenic substance in extracts active in inducing immunity was also tested by the following experiment.

Mice were immunized actively with M extracts prepared by heating living streptococci with N/20 HCl for 15 minutes in a boiling water bath.   The antigen was further purified by reprecipitating twice from saline solution with three to four volumes of 95 per cent ethyl alcohol.   A neutral solution of the antigen was filtered through a Berkefeld N filter; and the amount to be injected was calculated on the basis of the trichloroacetic acid precipitable fraction.   The antigen was distributed in tubes, each containing enough for one day's immunization, and dried from the frozen state on the Flosdorf-Mudd lyophile apparatus.

In Table VII are recorded the results of testing mice immunized with the extract.   One set of animals received 0.33 mg. of material, the same dosage as those recorded in Tables I and II.   Only 20 per cent of these survived an inoculation of 100 M.L.D. of the homologous strain S118.   In another set which was immunized with 6.6 mg., 60 per cent survived a similar inoculum.   A preliminary test with a similar M extract, but unfiltered, also showed 60 per cent survival when large immunizing doses were given for 4 weeks.

Preliminary Chemical Studies of the Antigenic Extract

Total nitrogen and phosphorus analyses on three typical extracts are given in Table VIII.   Since the high phosphorus content and the precipitability at pH 4.5 suggested that nucleic acid might be present,

spectroscopic examination of several preparations was made.[5] All showed an absorption spectrum characteristic of nucleic acid, that is, a wide band with maximal absorption at about 2,600 Å. Quantitative spectroscopic estimates, using yeast nucleic acid as a standard, in-

TABLE VI

Absorption Experiment

Passive Protection Test in Mice

Type 1 antibacterial serum		Culture: Type 1, strain S118			
		10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.
	cc.				
Unabsorbed	0.5	S	S	S	S
	0.25	S	S	S	S
	0.12	S	S	S	D 2 days
	0.06	S	D 3 days	D 2 days	D 12 "
	0.03	D 3 days	S	D 2 "	S
Absorbed with type 1, S118	0.5	D 1 day	D 3 days	D 1 day	D 1 day
extract: Lot 61 antigen	0.25	D 1 "	D 2 "	D 2 days	D 2 days
used for active and pas-	0.12	D 1 "	D 1 day	D 1 day	D 2 "
sive immunization tests	0.06	D 1 "	D 1 "	D 2 days	D 2 "
	0.03	D 1 "	D 1 "	D 2 "	D 1 day
Absorbed with type 1, S118	0.5	D 1 "	D 1 "	S	S
M extract, made with	0.25	D 1 "	D 2 days	D 2 days	D 2 days
N/20 HCl at 100°C.	0.12	D 1 "	D 1 day	D 2 "	D 5 "
	0.06	D 1 "	D 1 "	D 1 day	D 3 "
	0.03	D 1 "	D 1 "	D 2 days	D 3 "

Virulence controls: Inoculated with strain S118

20 mice inoculated with 10^{-6} cc.	6 mice inoculated with 10^{-7} cc.	6 mice inoculated with 10^{-8} cc.
10 mice D 1 day	3 mice D 2 days	6 mice S
8 " D 2 days	1 mouse D 5 "	
2 " S	2 mice S	

dicated that usually 25 to 30 per cent of the material in the extracts was nucleic acid. The biuret test and the Sakaguchi test for arginine were positive in high dilutions of the extracts. Since the relationship

[5] We are indebted to Dr. George Lavin for the spectroscopic determinations on these preparations.

TABLE VII

Active Protection Test in Mice
Immunized with M Extract* of Strain S118

Immunized mice: Immunity tested by inoculating with 10^{-6} cc. strain S118		
Mouse No.	Each mouse immunized with total dosage 0.33 mg. of extract	Each mouse immunized with total dosage 6.6 mg. of extract
1	D 1 day	D 1 day
2	D 1 "	D 1 "
3	D 1 "	D 2 days
4	D 2 days	D 4 "
5	D 4 "	S
6	D 4 "	S
7	D 4 "	S
8	D 4 "	S
9	S	S
10	S	S

Virulence controls: Inoculated with strain S118		
20 mice inoculated with 10^{-6} cc.	5 mice inoculated with 10^{-7} cc.	5 mice inoculated with 10^{-8} cc.
16 mice D 1 day 2 " D 2 days 1 mouse D 5 " 1 " D 12 "	2 mice D 1 day 3 " D 2 days	4 mice D 2 days 1 mouse S

Immunization was carried out in the same manner as with the mice reported in Table I, each set receiving four series of injections.

* Extract prepared by heating at 100°C. with $\text{N}/20$ HCl.

TABLE VIII

Chemical Analysis of Typical Fractions Used for Immunization

Lot No.	Extract from		Organisms heated at 56°C.	N	P	N precipitated by trichloroacetic acid	Nucleic acid
			min.	*per cent*	*per cent*	*per cent*	*per cent*
61	Strain S118	Type 1	15	16.73	3.97	52.06	33.3
62	" D58	" 3	15	15.60	3.69	50.61	6.9
65	" S118	" 1	5	16.25	3.40	58.72	30.2

of these findings to the constitution of the active agent in the extract is not certain, further work is being carried on in an attempt to clarify this point.

DISCUSSION

A substance has been obtained from extracts of group A hemolytic streptococci which induces active immunity in mice. On injection into rabbits it leads to the production of relatively type-specific antibody with which mice may be passively protected against infection with strains of the homologous type. Although the immunity in general was predominantly type-specific in nature, some non-type-specific reactions were also observed. It is important to realize that the methods employed for extracting the streptococci would probably not yield any single substance in a form approaching purity. This conception is strengthened by the previous work which suggests that the hemolytic streptococcus contains many proteins which are precipitable at a pH near 4.5 (2) and this idea is, moreover, confirmed by the cross reactions seen in precipitin tests with some of the antisera obtained from rabbits immunized with this material. Although the first experiment (Table I) indicated active protection against the homologous strain, the second experiment (Table II) showed that this was not strictly type-specific. This cross immunity may be explained by assuming either that it was induced by other antigens in the immunizing extracts having a broader specificity than the type-specific substance or that there are chemical and antigenic relationships among the type-specific substances themselves.

In the passive protection experiments, on the other hand, type-specific immunity was striking, and the indication of cross protection among types so slight that its existence is questionable. In the precipitin tests with the immune sera prepared in rabbits and used for passive protection tests in mice (Table V), both type specificity and cross reaction were observed. The cross reactions here are open to the same interpretation as in the experiment on active immunity. Although precipitin absorptions were not performed in the present experiments, previous work based on absorption experiments indicates that cross reactions observed in the precipitin test are probably due to an admixture of antigens in the extract used to immunize animals, and, correspondingly in the M extract used as reagents in the test tube.

In developing a method of extracting the antigen, different procedures were tried, and the extracts were compared in their ability to

produce immunity in mice. While the method used in the present experiments was the best of those tried, there are certain steps in the procedure which were used empirically. In the light of experience gained since this method was adopted, it may be that heat-killing and grinding the bacteria prior to extraction are not essential steps in obtaining the best antigens. We feel that it is important, however, to use young, actively growing cultures, prepared by the method described. The comparative experience in immunizing rabbits, which shows that large doses of antigenic extract did not induce as potent antisera as smaller doses of whole streptococci, makes us feel that the antigenicity of the active substance had been impaired by extraction. Possibly some refinement of these extraction methods or a different procedure may furnish an antigen with unimpaired or little diminished activity.

The nitrogen and phosphorus analyses of the extracts, the positive protein tests, and the spectroscopic analyses, as well as the behavior of the extracts in precipitating at pH 4.5, all suggest the presence of a nucleoprotein. It is impossible to say at present whether a nucleoprotein is the agent active in inducing the immunity observed, since the extracts were undoubtedly impure preparations. Conceivably a very small admixture of some other substance may have been responsible for the antigenic activity.

The antigen we have extracted cannot, as yet, be compared with the labile antigen described by Mudd and his coworkers,[1] since they have studied that fraction mainly in its ability to absorb precipitins, agglutinins, opsonins, and of protective antibodies from antibacterial serum, while we have confined ourselves principally to the production of active immunity in mice and protective antibodies in rabbits. Sevag, Lackman, and Smolens (18) have recently stated that the labile antigen is a nucleoprotein. While the active agent in the extracts described by us may be a nucleoprotein, it does not necessarily follow that it is the same as the labile antigen, since it is known that there are many nucleoproteins in the streptococcus (2), some of which are undoubtedly non-type-specific in nature.

The extract studied by Stamp and Hendry, however, is similar to the one described here, since both are made with N/10 HCl at 37°C. Although the subsequent method of purification is different, both ex-

tracts produce active immunity in mice against the homologous strain of streptococcus.

The active protection experiment in mice with the type-specific M substance extracted with N/20 HCl in a boiling water bath, shows that antigenic material is present, even though it is necessary to give larger amounts of it to obtain a degree of protection comparable to that elicited by the extract described in this paper. Both kinds of extracts react similarly in precipitin tests with specific immune sera, and both are also similar in the way they absorb the protective substance from immune sera. This last finding is at variance with the conclusions of Mudd and his collaborators, who were unable to absorb the protective substance from antibacterial sera with M substance prepared as described above.

The evidence presented, together with the general characteristics of the substance and manner of isolation, leads us to believe that the active principle in the extracts used for active and passive immunization in the present experiments is essentially similar to the active principle in the M extracts as formerly prepared, and probably also to the antigenic substances obtained by Stamp and Hendry, although different preparations exhibit varying degrees of purity and degradation from the native state. This substance, immunologically distinct for each serological type of group A hemolytic streptococcus, is probably the type-specific constituent in at least partially antigenic form.

SUMMARY

1. A substance extracted from group A hemolytic streptococcus is described, which induces active immunity in mice, and in rabbits gives rise to precipitins and to protective antibodies passively transferable to mice.

2. The active immunity in mice is principally type-specific, but some degree of non-type-specific immunity is also developed. The passively transferable protective antibodies are type-specific with only a slight suggestion of non-type specificity. In the precipitin test, the rabbit immune sera give both type-specific and non-type-specific reactions which have not been fully analyzed serologically.

3. Substances contained in the extract absorb the protective antibodies from the serum of rabbits immunized with whole hemolytic streptococci.

4. The most satisfactory method of extraction so far developed is fully described. Chemical tests on the material are consistent with the presence of protein and nucleic acid.

5. The type-specific M substance, prepared as previously described, was compared in some of its antigenic properties with the above mentioned substance. It was found capable of inducing active immunity in mice and of absorbing protective antibody from anti-bacterial immune serum in a manner qualitatively similar to that obtained with the preparations made by the newer methods.

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UTERINE ADENOMATA IN THE RABBIT

II. Homologous Transplantation Experiments

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Plates 28 and 29

The occurrence and course of spontaneous adenomata and adeno-carcinomata of the uterus in the rabbit and the successful transplantation of one of the tumors to animals of the same species were reported in a previous paper (1). Investigation of the transplanted tumor has been continued and extended in various directions with a view to determining the essential biological and pathological characteristics of the growth as compared with other transplantable tumors now used for experimental purposes.

Many spontaneous tumors have been reported in the rabbit but very few have been successfully transplanted to normal animals. With the exception of the Brown-Pearce epithelioma (2) all of the transplantable tumors have been sarcomata and with the further exception of Kato's sarcoma (3) all were lost before any extensive experimentation could be carried out. During the past year, three different types of spontaneous rabbit carcinomata, in addition to the uterine tumor, have been successfully transplanted and are being studied in a similar manner to supply a broader basis for comparison and as additional agents for the investigation of cancer problems. It has been found that the uterine tumor, designated as H31, is manifestly different from other transplanted rabbit tumors and possesses certain potentialities which render the growth a more advantageous medium for the study of certain phases of the tumor problem.

The object of the present paper is to report the results of several series of interrelated experiments including: first, serial transplanta-

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tion in normal rabbits; second, transplantation in rabbits with sponta-
neous tumors of the same nature; third, reinoculation of animals with a
growing transplanted tumor; fourth, reinoculation of animals which
proved refractory to an earlier attempt at transplantation and of
animals in which transplanted tumors had completely retrogressed and
finally, a series of experiments in which frozen and dried tumor
material was introduced into normal uteri.

While this report must be limited to results obtained with the
uterine tumor referred to above, comparative statements of results
are based in part upon experience with other tumors.   Details of these
experiments will be reported later.

Materials and Methods

The technique of transplantation employed in the present experiments has
been fully described elsewhere (1).   Whole fragments of tumor tissue measuring
approximately 1 mm. in diameter were used in transfers to the anterior chamber
of the eye, while testicular inoculations were made with 0.3 cc. of a thick cellular
emulsion.

The present report is based on a clinical and pathological investigation of the
tumor through 12 serial generations in the anterior chamber of the eye and 6
serial generations in the testicle.   Tumor tissue was transferred to the eyes of
23 normal males and 62 normal females while 95 males were used for testicular
inoculation.   The results of simultaneous inoculation of both testicles or of one
testicle and an eye were studied in 5 animals.   Transplants were also made in 8
females with spontaneous uterine tumors and 13 animals in which the tumor had
failed to grow were reinoculated after periods of 35 and 69 days following the
first inoculation.   In addition, transplants were made in 3 animals in which a
previously transplanted tumor was actively growing and in 13 animals in which
the previously transplanted tumor had completely retrogressed.

Six animals were used in an attempt to induce neoplasia with dried frozen
tumor material.   Fresh tumor tissue was obtained from the eye of an animal of
the 6th serial generation.   The tissue was immediately frozen in dry ice and dried
in a vacuum machine.   The resulting powder was emulsified in water and in-
jected directly into the uterine mucosa.   The animals were killed 42 days after
injection and sections of the mucosa were examined histologically.

Throughout the experiments particular attention was directed toward a thor-
ough gross and histological postmortem examination, not only to follow the
morphological characteristics of the tumor and to discover the presence or absence
of metastases but, also, to determine the condition of those organs altered in
spontaneous cases of the tumor.   Pituitary glands were fixed in Susa's solution
and stained by a modification of Mallory's aniline blue method, while routine

tissues were fixed in Petrunkevitch's solution and stained with hematoxylin and eosin.

Serial Transplantation into the Anterior Chamber

The results of serial transfer of tumor fragments into the anterior chamber of the eye are presented in Table I. Successful transplantaion was effected in 82.8 per cent of all the animals used but varied from 33.3 per cent to 100 per cent in different generations. In early

TABLE I

Anterior Chamber Series

Generation	Date of transfer	Number of animals used	Takes
	1937		*per cent*
1	Mar. 10	3	66.6
2a	May 18	3	33.3
2b	" 28	3	66.6
3	Aug. 10	6	33.3
4	Oct. 21	8	87.5
5	Nov. 29	8	87.5
	1938		
6	Jan. 13	7	100.0
7a	Feb. 24	6	83.3
7b	Mar. 3	6	50.0
8a	Apr. 5	3	100.0
8b	" 12	14	100.0
9	May 18	7	100.0
10	June 20	5	100.0
11	July 20	8	75.0
12	Sept. 21	6	100.0
Total......		93	82.8

transfers the tissue was obtained from transplants varying from 2 to 3 months in age while in later transfers the interval between transplantations rarely exceeded 1 month.

The age of the transplant undoubtedly played a part in the greater incidence of takes but there is also evidence that adaptation to the environment of the anterior chamber was of considerable importance in this respect. Two experiments bear on this point. In one experiment tissue fragments from a growth in the testicle representative of

the 7th generation of the tumor were transferred to the anterior chambers of 14 animals and resulted in 38.5 per cent of takes. In a second experiment the transfer of fragments of another testicular growth of the same generation into the eyes of 13 animals resulted in 41.6 per cent of takes. These results are similar to those observed in the first part of the series under discussion, despite the fact that the tumor had been successfully transplanted for 7 generations, and indicate that the high percentage of takes in the latter part of the series was influenced by adaptation to the environment of the anterior chamber, rather than by adaptation to transplantation in general.

The regular increase in the incidence of takes was interrupted in August, 1937, and in July, 1938. A similar reduction in the number of takes during these months has been noted in the study of other tumors propagated by different routes of inoculation and has occurred to a much greater degree in the testicular series of the present tumor.

Latent Period.—A gradual decline in the period required before growth of the transplanted fragments became apparent was observed throughout the first 5 generations but, even after the 5th transfer, definite evidence of growth could only rarely be detected in less than 1 month. Abruptly after the 6th transfer this period was cut to 14 days and while, at the present time, signs strongly indicative of growth may be found in a shorter period, an earlier positive diagnosis of growth cannot be made.

The first indication of survival is a pinkish color change in the transplanted fragment without any appreciable increase in its size. At the present time, this can often be detected by the 6th day. The color change appears simultaneously in all parts of the fragment and increases slightly in intensity throughout the following week. In contrast, fragments that fail to grow become dull white in color and opaque. In recent transfers, minute pin point pinkish-white areas are frequently observed in regions where formerly no trace of the transplant could be seen. Such areas have been interpreted as resulting from the growth of cells dislocated from the graft during its passage through the chamber.

An increase in the size of the transplant can usually be detected by continuous observation during the 2nd week. This is facilitated by the transplantation of fragments with irregular outlines so that small changes in a given locus can be readily perceived. Comparative drawings taken at daily intervals, in such cases, show a slight but definitely detectable tendency toward the rounding out of sharp, angular irregularities which is usually apparent by the 8th day. This process continues and at the time of vascularization, the irregular outlines have disappeared and the transplant is round or oval in shape.

Vascularization and Subsequent Growth.—The transferred tumor fragments

always become attached to a fixed part of the anterior chamber, usually the iris, within 12 hours of transplantation. Attachment occurs whether or not growth subsequently takes place and is evidently brought about by an exudative reaction on the part of the host. It is not associated with a connective tissue or vascular proliferation but is loose and may be disrupted by applying pressure along the corneal surface. Evidence of vascularization has not been detected before the 14th day and is frequently not observed before the 21st day. It should be noted, however, that stroma replacement and vascularization began along the area of attachment and the process may be of some duration before blood vessels can be seen in the gross on the exterior of the transplant.

In all but a single instance, fragments that have undergone the color change and increase in size previously described have eventually become vascularized. Vascularization is often delayed for as long as 3 weeks and in the interim the fragment may double its mass or remain without appreciable change. In exceptional cases, fragments have persisted without a detectable blood supply or increase in size for 2 months and then have become vascularized with subsequent rapid development into large tumors.

Vascularization is effected by a growth of vessels from the iris which permeate the graft in all directions and form a fine, complicated network about the growing edge. The appearance of the vascularized transplant is sufficiently characteristic to differentiate it from grafts of other tumors of different origin growing in the same environment. The tissue appears homogeneous and pale pinkish-white in color, except for the peripheral region which is of a deeper hue. Occasional minute greyish flecks are seen but the patterned arrangement of dark and lighter areas found in other growths is entirely absent. The tendency to form rounded masses with smooth clear-cut edges persists until infringement on the boundaries of the anterior chamber forces a change of shape.

Eventual Fate.—A large proportion of the animals used in these experiments were killed as soon as successful transplantation into the next serial generation became apparent, but others were held to determine the eventual fate of the growth.

The growth rate was increased after vascularization in all instances but varied in different generations and in individual animals. The period required for the complete filling of the anterior chamber varied from 45 to 105 days in animals of the same generation and, while the chamber was rarely filled in less than 60 days in the earlier transfers, complete replacement has been observed by the 20th day in recent generations. It should be noted, however, that in recent generations, growth invariably occurred in multiple foci and the resulting nodules coalesced so that the increase in growth rate was not as great as is suggested by these time relations.

After filling the anterior chamber, the growth underwent regressive changes in approximately 70 per cent of cases. The tissue became brown, granular and opaque and, eventually was entirely resorbed leaving no permanent damage other than large corneal scars and occasional synechiae. On the other hand, in the remaining 30 per cent of cases, growth continued, the cornea was invaded and the tumor protruded externally as a large fungating mass. Animals of this class have been killed for humane reasons, and hence there is no telling what their eventual fate would have been. Autopsy revealed the presence of metastases in two instances. One animal had been killed on the 184th day after transplantation and secondary growths were found in the regional lymph nodes, the lungs, the pancreas and the left ovary. In another animal killed on the 161st day, the regional nodes alone were involved.

As a rule, the more rapidly growing transplants undergo regression after filling the chamber, while those with a slower growth rate tend to invade and to extend to the outside. It seems probable that the regressive changes may be a direct result of an increased intraocular pressure incident to rapidly expanding growth in a confined space and leading to a progressive diminution of blood supply. The fact that, in the normal course of events, regression has never been observed before the entire chamber is filled is also suggestive in this respect. It is of interest in this connection that surgical interference with removal of a small part of the graft is almost invariably followed by regression.

No attempt was made in the present series of experiments to select animals to test the effect of different constitutional factors on the susceptibility to transplantation or on the eventual fate of the growth. Hybrids were used, for the most part, and no indication of breed differences can be obtained from the data. An analysis of the results on an age basis shows that while no significant variation in the susceptibility to transplantation occurred, the transplants grew more rapidly in young animals and after replacing the anterior chamber, regression rather than continued extensive growth was the rule. The percentage of takes was the same in males and in females and the subsequent fate of the tumor was not altered by the sex of the animal.

Histological Examination.—Histologically, the transplants obtained from animals of early serial generations showed an approximate duplication of the characteristics of the parental tumor (Fig. 1). There was an abortive attempt at the formation of acini which for the most part were composed of solid cellular masses and were without a lumen. Mitotic figures were not numerous and degenerative changes were rarely found. The stroma was abundant and myxoid in character.

In later generations, growth was more atypical and anaplastic (Fig. 2).

A tendency to form rounded masses was still apparent, particularly along the advancing edge of the tumor, but in other regions individual masses had coalesced to form solid sheets. In such regions the frequent occurrence of round areas of necrosis surrounded by concentrically arranged epithelial cells indicated the manner of growth. Stroma was sparse and poorly differentiated. Large cellular regions were separated by fibrous connective tissue extensively invaded by epithelial cells which formed abortive acini in some areas but grew, for the most part, in short strands and columns giving the tissue a marked resemblance to sections of scirrhous carcinoma. The number of mitotic figures and the amount of necrosis were directly proportional to the rate of growth observed clinically.

Necrosis dominated the picture in animals killed while the tumor was undergoing clinical regression. Large circumscribed necrotic areas were distributed focally throughout the cellular regions and in older growths these regions were completely necrotic except for a narrow rim of intact cells surrounding vascular channels. On the other hand, epithelial cells enmeshed in fibrous tissue remained intact for a longer period of time and the eventual disintegrative changes proceeded through karyorrhexis rather than karyolysis as in the more cellular areas.

All structures of the anterior chamber with the exception of the lens were invaded and destroyed in the large fungating types of growth. The posterior chamber was frequently involved and was occasionally found filled with tumor, but extension through the sclera to retro-orbital tissues has not been observed.

Metastases were less cellular in structure than the primary growth and the connective tissue reaction was more marked (Figs. 3 and 4). The proliferation of fibrous connective tissue was so intense in the lung, particularly near the pleural surface, that serial sections were frequently required before the essential epithelial elements could be found. The resemblance to scirrhous carcinoma was striking in these regions but in other organs parenchymal cells were grouped in atypical acinar arrangement and the stromal relations approximated those of the primary growth.

Serial Transplantation in the Testicle

Transplantation of the tumor into the testicle was successfully carried out from the 3rd, 4th and 5th serial eye generations and the growth obtained from the 4th eye generation has been propagated by continued serial transfer in two different lines of animals.

The results of serial transfer by this route of inoculation are pre-

sented in Table II. Growth occurred in only 41.05 per cent of the animals used and the success of inoculation was extremely irregular in the different serial generations. 100 per cent of takes was obtained at one transfer while other transfers resulted in complete failure. The more successful transfers were made during the fall and winter months

TABLE II

Testicular Series

	Testicular generation	Serial generation of tumor	Date of transfer	Number of animals used	Takes
			1937		*per cent*
Series A	1	4	Oct. 21	2	50.0
	2	5	*1938* Mar. 3	4	75.0
Series B	1	5	*1937* Nov. 29	3	66.6
	2	6	*1938* Jan. 12	8	25.0
	3	7	Mar. 3	3	100.0
	4	8	Apr. 5	9	66.6
	5	9	June 13	4	75.0
	6	10	July 21	8	25.0
	6	10	Aug. 22	7	00.0
Series B₂	3	7	Feb. 16	11	54.5
	3	7	Mar. 3	10	50.0
	4	8	Apr. 4	12	25.0
	5	9	May 28	5	00.0
	5	9	July 13	4	00.0
Series C	1	6	Feb. 4	5	80.0
Total............				95	41.05

and the largest per cent of takes resulted from inoculations made in February and early March. On the other hand, the failures occurred in the late spring and summer.

A distinction between the effects of adaptation and of season on the behavior of the testicular transplants is extremely difficult in the present series. Extreme seasonal variations are known to occur and

the present series of experiments were undertaken at different seasons. Thus, while the success of the first generation transfers appears to be directly related to the length of time that the tissue used had previously been serially transplanted in the eye, there is no indication in the data as to whether season or adaptation to transplantation was the determining influence. It is evident, however, that the percentage of takes in subsequent testicular generations was not increased by continued passage in that organ.

The percentage of takes was not consistently increased by shortening the interval between transfers but here again the influence of season may have been operative. On the other hand, the latent periods in the first generation transfers averaged 30 days irrespective of the season in which inoculations were made. Moreover, this period decreased to 14 days in subsequent generations and was not altered with changes of season.

The rate of growth varied within wide extremes but the variations were more marked between animals of different genetic constitution in the same generation than between similar animals in different generations. In some animals, the growing nodule never progressed beyond the size of a pea and was morphologically distinguished by an intense connective tissue proliferation and an acinar arrangement of epithelial cells (Fig. 5). On the other hand, the majority of animals developed multiple nodules which grew rapidly and completely replaced the testicle by the 40th day after inoculation. Histologically, such tumors resembled the rapidly growing transplants in the anterior chamber and were characterized by large confluent cellular masses with centrally placed areas of necrosis (Fig. 6). Growth was both expansive and extensive in character, destroying testicular parenchyma both by pressure and by active infiltration.

Infiltrative growth was limited to the testicular parenchyma for a long period of time and extension to the tunica vaginalis or to the spermatic cord was not observed before the 160th day. Expansive growth continued and eventually with encroachment on the blood supply, fluctuating necrotic areas appeared. Occasionally the entire testicle was converted into a sac distended with black fluid necrotic material, but even in such instances active nodules of growth were found on histological examination. In the majority of cases, however, such degenerative changes were limited to small areas and connective tissue replacement rather than necrosis characterized the older growths. The connective tissue was, in turn, invaded by tumor cells growing in strands and in isolated acinar groups. Growths of this type reached a large size and after the 160th day the testicle frequently measured 7 × 5 cm. and was characterized clinically by a firm nodularity. The eventual outcome of such cases is not known. One animal killed

on the 216th day showed fibrosis of the testicle with a complete destruction of all tumor cells, while the growth in another animal held for 240 days is still increasing in size.

Metastases have been found in three instances.   The lymph nodes of the mesentery of the large intestine were involved in one animal killed on the 90th day and in a second animal killed on the 113th day which, in addition, showed a large metastatic nodule in the substance of the diaphragm.   The third animal was killed on the 233rd day and secondary growths were found in nearly all organs of the body (Fig. 7). Microscopically the structure of the secondary growths was similar to that of metastases arising from transplants in the anterior chamber.

Simultaneous Transfers to Different Sites

Bilateral growths have been obtained from the simultaneous inoculation of both testicles in the same frequency with which inoculations into a single testicle have proved successful.   Simultaneous transfers to the testicle and to the eye have resulted in growth in the eye alone in three instances and in growth in both locations in two instances. It is of interest that in one of the latter cases a metastatic growth was found at autopsy in a lymph node of the anterior triangle of the neck, while metastases were not found in the drainage area of the testicle.

Reinoculation of Refractory Animals

Transplantation of the tumor into the testicles following a primary unsuccessful inoculation has been attempted in 13 animals.   The same testicle was used in the second attempt throughout the experiments. Control inoculations were made and gave a high percentage of takes.

Reinoculation of 12 of the animals was performed 35 days after the primary failure and was unsuccessful in all instances.   On the other hand, reinoculation was delayed for 69 days in one animal and resulted in a take.

Transplantation into the Anterior Chamber of Animals with Spontaneous Uterine Tumors

The transfer of tumor fragments into the anterior chambers of 8 animals bearing spontaneous tumors of the same nature resulted in 5 takes, an incidence of about 60 per cent in contrast to an incidence of

approximately 80 per cent in normal animals. The transfers were made coincidently with the 1st, 2nd, 4th, 5th and 9th serial transplantations previously described and tumor material of the same derivation was transferred to both types of animals, but the incidence of takes in tumor bearing animals was always less than in normal animals of the same generation and did not increase with the incidence of takes in normal animals.

The latent period was of similar duration in both types of animals and the subsequent progress of growth in three of the tumor bearing animals was comparable with that observed in normal members of the same generation. One animal was killed on the 60th day and the tumor which occupied approximately 2/3 of the chamber showed the usual histological characteristics noted at that period. In another instance, the growth had replaced the anterior chamber on the 83rd day and had invaded the posterior chamber when the animal was killed on the 141st day. The anterior chamber of the third animal was completely filled on the 52nd day but at autopsy on the 105th day the tumor had almost entirely disappeared and the remaining portion was largely necrotic.

The progress of growth in the remaining two animals differed radically from that noted in normal animals. The growth rate was extremely slow in both instances and the transplants had no more than doubled in size after 130 days. At this time the animals were killed. Microscopic examination showed that the growth in one animal was almost entirely necrotic while the tumor in the other animal was characterized by an abundant myxoid stroma with epithelial cells arranged in well defined acinar formations (Fig. 8), despite the fact that the tumor in normal animals of the same generation showed an almost solid cellular structure with a minimum of supporting elements.

The success of transplantation in these instances appeared to be directly proportional to the size and age of the spontaneous tumors as judged by morphological examination and by a study of the breeding histories of the affected animals. The transfer of fragments to animals with small, early tumors resulted in three failures and two small slow growing nodules, while the inoculation of animals with large, older tumors was followed by takes and rapid growth in all cases.

Other Reinoculation Experiments

A series of experiments was designed with the view of obtaining more information regarding the effect of a growing tumor on the transplantation of other tumors of the same and of diverse nature. The performance of the experiments has been delayed because of the low

incidence of takes in the testicle during the summer months, which forced a temporary discontinuance of the series. While relatively few of the experiments have been completed and the available data do not justify conclusions, the results so far obtained seem to be of sufficient interest to warrant recording.

The inoculation of the left testicle with tumor material obtained from the right testicle after its removal from the body has been attempted in two instances. In one, the right testicle was removed 35 days after a successful transfer and the immediate inoculation of the left testicle resulted in a take. The subsequent growth of the nodule in the left testicle was similar in all respects to that previously observed in the right. In the second instance, on the other hand, the affected testicle was not removed until the 135th day and inoculation of the remaining testicle was not followed by growth. Control inoculations resulted in 57.1 per cent of takes in the first instance and in 100 per cent of takes in the second instance.

An attempt to inoculate the testicle with material obtained from biopsy of the eye 66 days after transplantation into that organ was likewise unsuccessful although the inoculated tissue grew in the testicles of all of the control animals.

In another experiment, the tumor was successfully transplanted to the anterior chamber of the right eyes of 13 young animals. The chambers were eventually filled. by the growth which subsequently underwent complete regression and left extensive corneal scars. 167 days after the first transplantation, fragments of an actively growing tumor derived from the eye of a member of the 11th serial generation were transferred to the anterior chamber of the left eyes of the recovered animals and 8 controls were inoculated at the same time. Growth resulted in all of the controls but in no instance did a take occur in the reinoculated animals.

Postmortem Examination

A detailed postmortem examination was made of all animals included in these experiments, and in view of the changes found in animals bearing spontaneous uterine tumors, particular attention was directed toward the endocrine system. In the majority of cases, the organs were not pathologically altered and lesions that were found

vere traceable to disorders common in the general animal population.
[i]n no instance were changes comparable to those observed in animals
bearing spontaneous uterine tumors found in animals bearing trans-
planted uterine tumors.

Inoculation with Dried Frozen Tumor Material

A watery emulsion of dried frozen tumor material was inoculated,
at laparotomy, into the uterine mucosa of 3 virgin females and 3 multi-
parae. The animals were killed 42 days after inoculation and the
uterine mucosa was serially sectioned. Microscopic examination of
these sections and of sections from other organs showed no alteration
from normal. Controls inoculated with the living tumor, on the other
hand, gave 100 per cent of takes.

DISCUSSION

In the report dealing with spontaneous tumors of the uterus, especial
emphasis was placed upon the constant occurrence of certain endo-
crine changes most evident in the pituitary, suprarenals and thyroid.
In brief, the alterations in the pituitary were productive in nature
while those in the thyroid and suprarenals were retrogressive. These
alterations were present from the earliest stages of tumor development
and were also found, before histological evidence of neoplasia, in older
animals of the tumor line. This, in itself, suggests the possibility of an
antecedent change in the endocrine mechanism which had some
bearing on the eventual development of the neoplastic process. This
suggested relation is supported by the fact that the conditions observed
in animals with spontaneous tumors bear a striking resemblance to
those in animals subjected to long continued treatment with estrone,
and from this it was inferred that spontaneous tumor development
might represent a natural analogue to the experimental production of
neoplasia with such substances.

The point to be emphasized in the present discussion is that,
despite the fact that the uterine tumor under consideration has been
successfully transplanted into more than 150 rabbits and has grown
progressively for periods up to 11 months, none of these animals has
shown changes at autopsy which bore the slightest resemblance
to those constantly present in animals with spontaneous tumors. It is

apparent, therefore, that the endocrinological changes are not produced by the continued growth of neoplastic cells. It is also clear that the constitutional status associated with the endocrine changes is not an essential factor in the continued growth of neoplastic cells. In fact, the failure of transplanted tumors to grow actively or to grow at all in animals with early spontaneous tumors suggests that initially there is a contrary or inhibitory influence associated with such changes. It would appear then that the endocrinological conditions referred to were concerned with the initiation of neoplasia and that in the study of this tumor there are, as usual, two distinct problems to be considered, namely: the initiation of neoplasia and the continued multiplication and growth of neoplastic cells.

Investigation of the first problem is being carried out from the point of view of an exogenous as well as of an endogenous origin. Experiments based on the suggestion that the endocrine changes were associated with the initiation of neoplasia have been most encouraging and will be reported at a later date. On the other hand, attempts to demonstrate a causative agent of the nature of a filterable virus have so far been unsuccessful. Neoplasia was not initiated by the introduction of dried frozen material into the uterine mucosa. It is obvious, however, that numerous experiments of this character are necessary before a conclusion is warranted and such experiments are being continued.

The growth characteristics of the tumor have been studied in the eye and in the testicle. Both of these sites have been used by other workers in the investigation of other tumors. The testicle came into prominence following the work of Brown and Pearce with the transplantable epithelioma. They also used the anterior chamber in early experiments and reported a uniform series of takes with rapid growth to large tumors which, however, did not metastasize during the period of observation. A single case of spontaneous regression was noted (4). A sarcoma of the lower jaw had previously been successfully transferred to the anterior chamber by Schultze (5) and Happe (6). Their general results were in agreement with those of the present study but a further investigation of the characteristics and potentialities of the chamber as an inoculation site does not appear to have been carried out. The anterior chamber has also been successfully used in homol-

ogous transplantation experiments with tumors of other species (7) and was employed in many early heterologous transplantation attempts without apparent success.

While the susceptibility of the anterior chamber to tumor transplantation appears to be well recognized, it has not been widely used as an inoculation site. Certain features associated with its susceptibility and the characteristics of the resulting growth suggest, however, that a more general use might be of advantage. A comparison of the frequency of takes of the uterine tumor in the testicle and in the eye, as in Table III, brings out a number of points of interest. The relatively unimportant reduction in the number of takes in the anterior chamber during the summer months as compared with the complete failure of testicular inoculation suggests that this method of

TABLE III

A Comparison of the Percentage of Takes Resulting from Transfer to the Anterior Chamber and to the Testicle throughout the Year

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Anterior chamber....	100	83.3	55.5	100	76.9	100	75	33.3	100	87.5	87.5
Testicle	25	62.5	64.7	42.8	0	37.5	25	0	0	50.0	66.6

transfer might prove of great value in the maintenance of other tumors which are so frequently lost in testicular and subcutaneous series during this season.

In the series as a whole, the number of takes resulting from transfer to the anterior chamber was twice as great as followed inoculation into the testicle. This finding is in line with the fact that in no instance were first generation transfers from a spontaneous tumor to the testicle successful and that takes in the testicle were not obtained until the tumor had been passed through three generations of animals by serial eye transfer. While the cellular damage incident to the preparation of an emulsion may have been of influence in the lower frequency of takes in the testicle, the fact that 100 per cent of takes occurred at some transfers without modification of the technique indicates that this was not an important factor. It seems more prob-

able that the greater success in the anterior chamber is related to the slower reaction of the tissues of the eye to the presence of the transplanted fragment. The fragment grows for a longer period of time in the manner of a tissue culture and a degree of adaptation results before the occurrence of a foreign body reaction with the intimate contact between the cells of the host and the graft that determines its immediate fate.

Transplantation into the anterior chamber offers the further advantage of continuous visual examination of the graft. The effects of various procedures can be watched and the rate of growth can be measured easily with a pair of calipers. Metastasis may occur earlier and with greater frequency from testicular growths but the continued life of the animal is an asset in certain types of experiment. On the other hand, there are distinct disadvantages associated with ocular transfers. The anterior chamber is a small confined space surrounded by relatively inelastic tissues and the increased pressure which follows rapid growth may lead to necrosis and regression of the tumor. If the cornea is invaded or ruptured, the external extension of the tumor produces an unsightly fungating mass and trauma may lead to severe hemorrhage or infection. The great advantage of this method of transplantation lies in the relative ease with which first generation transfers from a spontaneous tumor can be effected. During the past four years more than 140 spontaneous rabbit tumors representative of 16 different types of growth have been observed in this laboratory. Attempts were made to transplant the different types of growth and until recently the testicular and subcutaneous routes were almost exclusively employed. It is significant that while these methods of inoculation failed in every instance, four out of five attempts to transfer by means of the anterior chamber proved successful.

It is not known whether the lowered incidence of takes during the summer months, particularly in the testicle, is related to an increased resistance of the animals or a decreased activity of the tumor cells. There is evidence, however, that even in winter months, periods occur during which an animal may be refractory to inoculation and it is conceivable that meteorological conditions more prevalent during the summer may bring about a refractory phase of widespread occurrence.

The spontaneous and.the transplanted tumors show many comparable characteristics. In both abnormal and normal hosts, the tumors progress slowly, growth is at first expansive and later infiltrative, and metastasis is a late occurrence. One characteristic of especial interest is shared by the transplanted tumor and by metastases of the spontaneous tumor. In both, there is evidence of an ability of the neoplastic cells to respond to different environmental conditions with an alteration in the degree of differentiation. Thus, in certain situations, metastatic cells grow in a well formed acinar arrangement and appear to be further differentiated than the cells of the primary growth. In like manner, the transplantation of a cellular, poorly differentiated tumor into animals of a special genetic constitution is followed by more highly differentiated growth with the formation of more or less typical structures.

In other respects the transplanted tumor behaves in a different manner and it should be emphasized that the behavior of neoplastic cells in normal animals is not a reflection of their behavior in the primary host where their activity may be influenced by an altered endocrinological status as well as by the presence of other growing neoplastic cells.

The observation has been made repeatedly by workers with some other tumors that, following successful transplantation, a phase occurs during which the animals are refractory to further inoculation of the same growth (8). In the present instance, it was also found that reinoculation gave negative results after continued growth of the transplanted tumor. Further conclusions cannot be drawn from the results obtained to date, but certain findings in regard to the refractory phase are of interest from the point of view of discussion.

Despite opinions to the contrary, it appears to be fairly clear that the refractory phase is brought about by the continued presence and growth of neoplastic cells, rather than by the absorption of products resulting from regressive changes in the tumor. The duration of the refractory phase in experimental animals in which the tumor under discussion had completely regressed was demonstrated to exceed 5 months. On the other hand, metastasis has occurred in other animals 3 months after transplantation. There is some evidence, there-

fore, that while growing neoplastic cells may bring about a refractory period, their continued presence and growth in the body may shorten the duration of this period.

The presence of a similar refractory phase in animals with spontaneous growths has not been satisfactorily demonstrated. It is generally believed that grafts of a spontaneous tumor are more apt to be successful if placed in another region of the same animal than if transferred to a normal animal and, in early experiments with the present tumor, it was found that subcutaneous autografts almost invariably grew while subcutaneous transfers to normal animals were uniformly unsuccessful. In these experiments, the growth had been present in the spontaneous host for more than a year before auto-inoculation was attempted and it is apparent that at this period of tumor development the animals were not refractory.

A determination of the susceptibility of animals in earlier stages of tumor growth to the transplantation of a malignant tumor of the same nature is a more difficult problem owing to the necessity of assembling a sufficient number of suitable animals as well as of determining the age of the spontaneous tumors. It should also be emphasized that in this type of experiment, in contrast to the experiments cited above, autogenous tumor material cannot be used inasmuch as in the early stages it represents benign rather than malignant neoplasia.

The occurrence of a considerable number of spontaneous uterine growths, the age of which could be determined with fair accuracy, offered a unique opportunity for study of this problem, and tumor material derived from various eye generations was transferred to the eyes of animals bearing spontaneous growths in different stages of development. A series large enough to provide significant results has not been tested and the experiment is being continued as more tumor bearing animals become available. The results are not conclusive for this reason but, while it must be borne in mind that further tests may give rise to contradictory findings, the present trend is of sufficient interest to warrant some consideration.

The uniformity of takes in animals with old tumors is in agreement with expectations based on experiments with autografts. On the other hand, the complete failures and the small slow growing nodules arising from transfers to animals with early spontaneous tumors are

more in line with the results obtained from the reinoculation of animals bearing transplanted tumors and may indicate the existence of a similar refractory phase. Additional evidence suggesting the presence of a growth inhibiting influence at this period is given by the fact that while neoplastic cells are present in the blood stream at early stages of tumor development, they fail to gain a foothold and grow until late in its course.

SUMMARY

The behavior of a transplanted adenocarcinoma of the uterus of a rabbit has been studied through 12 serial generations in the anterior chamber of the eye and 6 serial generations in the testicle. The transplanted tumor is characterized by slow growth which is at first expansive and later invasive, by an ability to form more or less differentiated structures in response to different environmental conditions and by late metastasis. The endocrinological changes that distinguish animals bearing the spontaneous tumor do not occur in animals bearing the transplanted tumor.

Various experiments were undertaken in an attempt to discover the nature of the factors determining the characteristics of the spontaneous and of the transplanted tumor. It was found that successful transplantation was followed by a phase during which animals were refractory to reinoculation. The results of transplantation into the eyes of animals with spontaneous tumors suggested the existence of a similar phase during the early development of the tumor but the number of observations was not sufficiently numerous to warrant definite conclusions.

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EXPLANATION OF PLATES

Hematoxylin and eosin was the stain employed throughout.

PLATE 28

FIG. 1. Section of a transplant in the anterior chamber of the eye resulting from the 2nd serial transfer. Stroma is abundant and epithelial elements tend to grow in abortive acinar formations.   × 67.

FIG. 2. Section of a transplant in the anterior chamber resulting from the 6th serial eye transfer. In contrast to the previous figure, stroma is sparse and epithelial cells are arranged in large rounded masses which frequently show necrotic centers. × 67.

FIG. 3. Section of a metastatic growth in the ovary derived from a transplant in the anterior chamber.   × 67.

FIG. 4. Section of a metastatic growth in the pancreas derived from a transplant in the anterior chamber.   × 40.5.
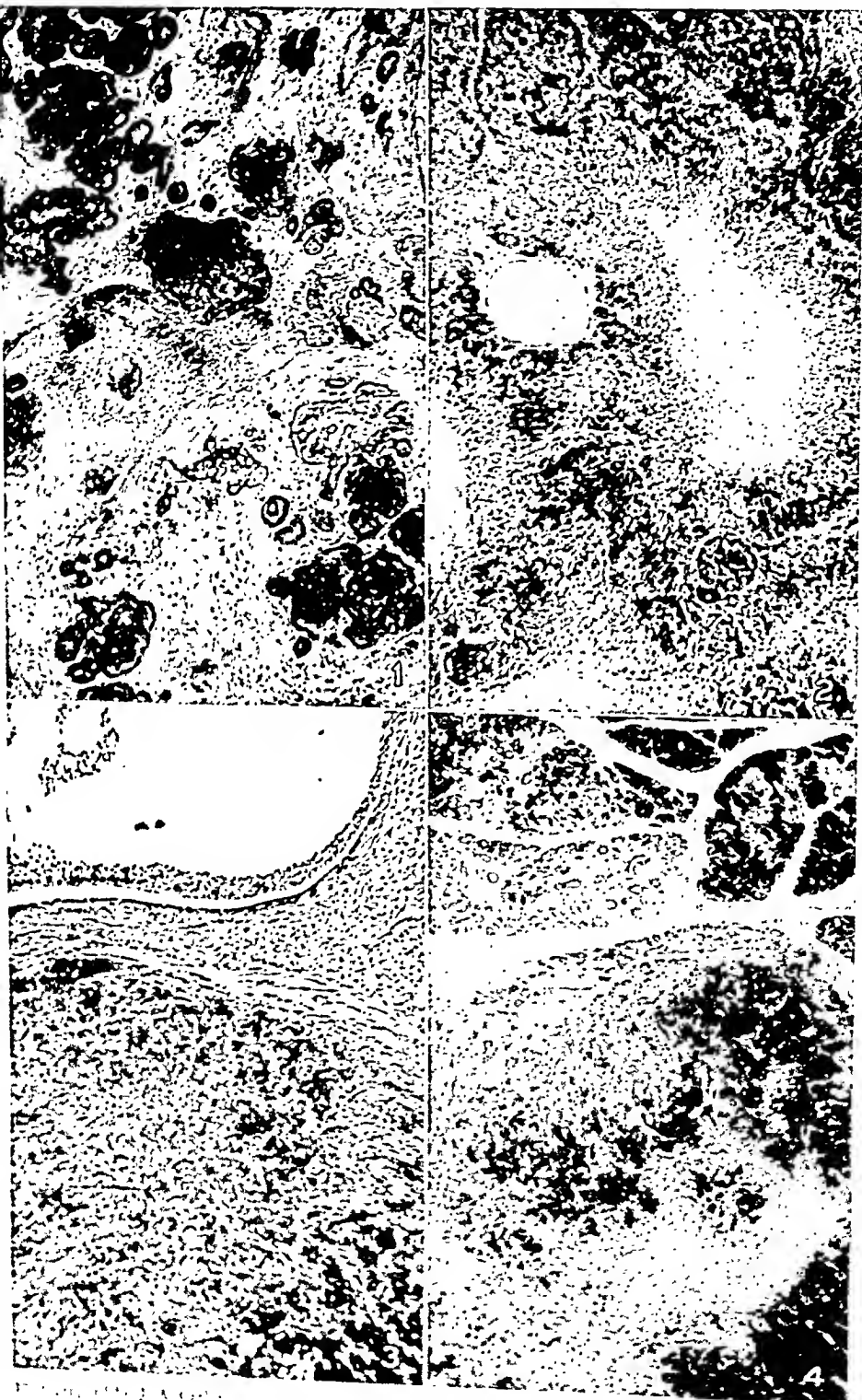
PLATE 29

FIG. 5. Section of a transplanted tumor in the testicle of a Himalayan rabbit. Stroma is abundant and epithelial cells grow in acinar-like groups. × 67.

FIG. 6. Section of a transplant in the testicle of a hybrid rabbit showing the characteristic histological features found in the majority of testicular grafts. In contrast to the appearance of the tumor in Himalayan animals, stroma is scanty and the arrangement of epithelial cells is similar to that found in rapidly growing eye transplants. × 67.

FIG. 7. Section of a metastatic growth in the lung derived from a testicular transplant. × 67.

FIG. 8. Section of a transplant in the eye of a rabbit bearing an early spontaneous uterine tumor of the same nature. Stroma is abundant and epithelial cells are arranged in well defined acinar formations. The appearance of control transplants in normal animals is shown in Fig. 2. × 375.
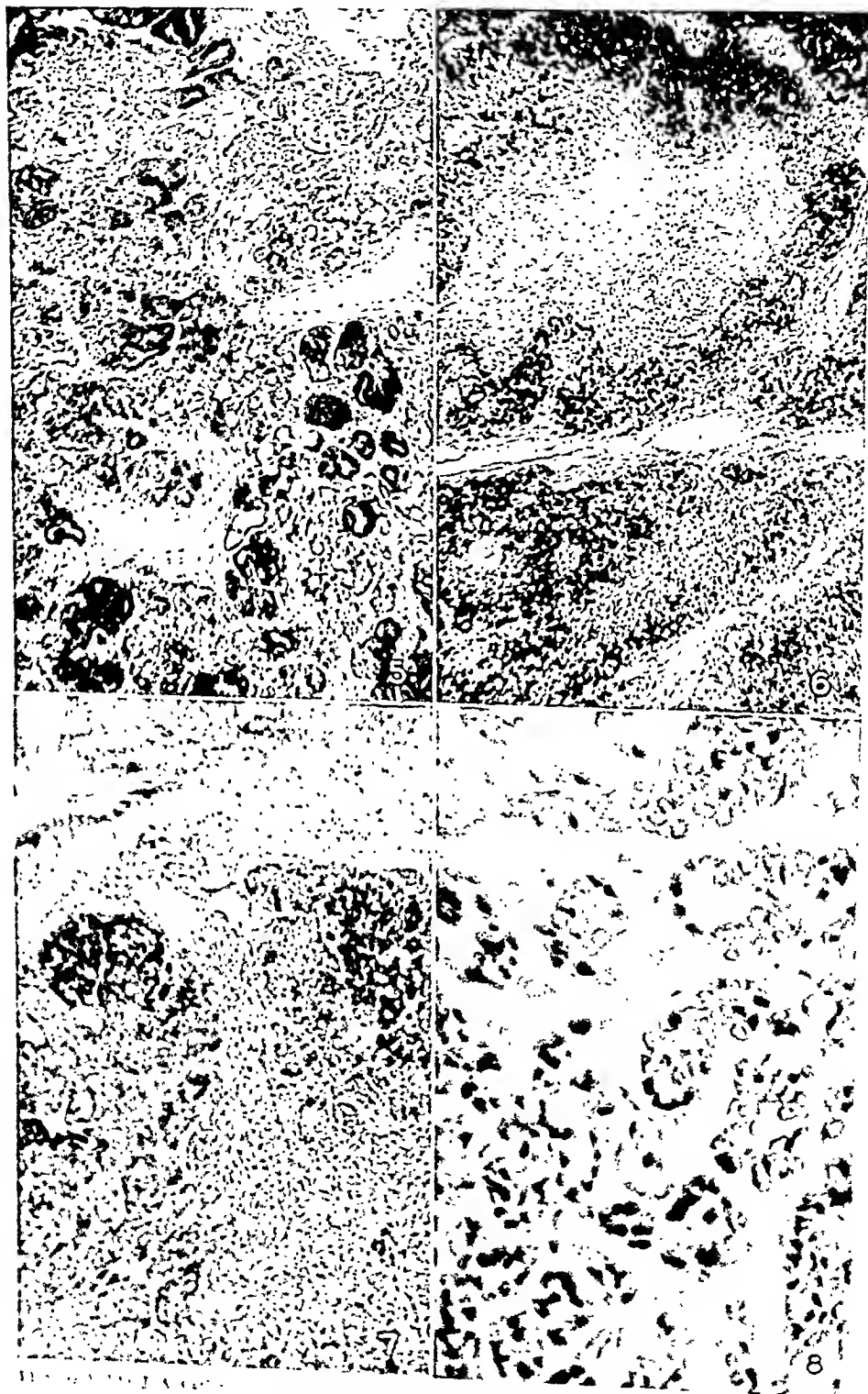
ORGAN WORK AND ORGAN WEIGHT*

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In 1924 Huxley (1) noted that there was frequently a constant relation between the relative rates of growth of the body and its parts, and in 1932 he assembled many examples of this constancy throughout the whole biological field (2). We have made use of this observation in examining data on the organ weights and body weights of a considerable number of albino rats that we had used as controls for experimental work.

EXPERIMENTAL

As will appear from the experimental data to be given, organ weight may fluctuate between wide limits when certain conditions are not kept constant, so it is important to specify the particular conditions under which these control animals were kept.

The food was given *ad libitum* and contained 18 per cent of protein. It was an air-dry mixture containing 10 per cent unpurified casein, 73 per cent corn meal, 10 per cent linseed meal, 2 per cent alfalfa, 3 per cent sardine oil, 1.5 per cent bone ash, and 0.5 per cent sodium chloride. The temperature of the room in which the rats were kept averaged 20°C. and was regulated to prevent any marked fall so that it was rarely less than 18°C., though on warm days it occasionally rose for a few hours appreciably above 20°C. in spite of a fan that kept a current of air constantly moving through the room. The rats were housed in groups of six in cages 17 × 12 × 12 inches. The colony came from the Slonaker strain from which the Wistar Institute colony was derived and for 12 years their ancestors had lived under conditions closely similar to those we have defined. They were all controls for experiments on the compensatory hypertrophy of the testicle, suprarenal, ovary, and kidney and had all undergone a sham operation in which these organs were exposed but not removed. The operations were performed on rats of exactly 30, 70, 110, and 220 days of age, and in each age division groups

were killed for measurement of organ weight 2, 5, 10, 20, and 40 days after operation. When the organ weight measurements were arranged in accordance with age instead of body weight it was possible to measure the effect of operation by finding the deviation from the predicted organ weights or from smoothed curves of the testes, female suprarenal, and ovary. But with the exception of the suprarenal gland no significant deviations were found. It should be noted, however, that the duration of ether anesthesia was very short and that the operation itself was over within about 2 minutes. The experiments that will be given were car-

TABLE I

Males							Females					
Number of rats	Body weight	Heart	Kidneys	Liver*	Supra-renals	Testes	Number of rats	Body weight	Heart	Kid-neys	Supra-renals	Ova-ries
	gm.	mg.	mg.	mg.	mg.	mg.		gm.	mg.	mg.	mg.	mg.
75	41-60	238	508	2305	13.90	358	69	41-60	240	516	14.50	21.0
69	-80	309	626		17.46	494	79	-80	298	614	17.94	24.6
54	-100	365	742		19.06	800	34	-100	363	762	22.50	31.6
46	-120	445	912		21.82	1064	32	-120	429	892	30.54	45.6
39	-140	498	1000		21.02	1364	82	-140	492	976	39.84	73.0
37	-160	554	1112		23.76	1598	134	-160	542	1068	44.74	80.6
77	-180	610	1252	7240	25.12	2024	157	-180	592	1116	48.36	87.4
79	-200	670	1350	7610	26.66	2326	127	-200	649	1222	48.74	90.2
53	-220	715	1448	8060	28.56	2416	63	-220	696	1300	48.76	84.6
56	-240	755	1492	8770	29.20	2590	20	-240	734	1370	48.70	85.2
45	-260	780	1590	8840	31.60	2654						
39	-280	841	1674	9830	32.70	2626						
51	-300	885	1774		33.36	2650						
39	-320	936	1844		34.90	2880						
36	-340	968	1900		35.26	2912						

* The liver weight measurements were made on another series of 229 rats kept under identical conditions except that there was no operation. More extensive data than we possess might reveal a sex difference in liver weight analogous to the sex difference in kidney weight but our present observations on female rat livers indicate that, if present, any such difference must be small.

ried out during the period over which these control observations were being made so that the possibility of change in the colony itself was excluded. Some of the measurements of the protein content of the liver have been already published in papers concerned with protein metabolism that are referred to in this paper, and the method used and the conditions observed are there described in detail.

The average organ weights arranged in accordance with body weight are given in Table I.

If the ratio between the rate of growth of the body and the rate of growth of any organ is constant, a plot of the logarithm of organ weight against the logarithm of body weight forms a straight line. When this is done for the data in Table I it is found that the organs fall into two classes, those whose logs fall along straight lines and those whose logs form sigmoid curves. In the latter class fall the testicle, ovary, and ♀ suprarenal, organs that for a short time during puberty undergo a marked growth acceleration. In the other organs, including those in which we are at present interested, there is an approximate constancy in the relative rates of growth of organ and body since the lines are straight. (See Figs. 1 and 2.)

The practical advantage of this straight line relationship is that it makes it easy to derive formulas with which organ weight may be predicted from body weight with considerably greater precision than is given by any of the other methods we have tried. Those made use of in this paper are given in Table II.

The slopes of the lines in Fig. 1 are determined by the method of least squares from the logarithms of the measurements given in Table I. These slopes give the ratios between the relative rates of increase of organ and body weight and are the powers of the body weight appropriate for each organ. Thus the heart weight varies as the (body weight)$^{0.750}$, indicating that the heart increases at 0.75 times the rate at which the body weight increases. The powers of the body weights were then calculated in numbers and the slopes of the lines obtained when the actual organ weight measurements were plotted against these numbers were determined by the method of least squares. The formula is now in the form organ weight = $a \times$ body weight$^n \pm b$. The constant a is the slope. The constant b, as Hall (3) has pointed out, is required because an extrapolation of the line does not cross the ordinate at zero. Actual observations from the body weight at birth show that the relationships given by the formulas do not hold below a body weight of 40 gm. and in the control observations given here the smallest animals had been weaned and had subsisted wholly on the control diet for at least a week. This b constant = average organ weight − ($a \times$ average body weight). To save the time required for calculation tables giving predicted organ weights for every gram change in body weight were constructed from large graphs.

The prediction errors given in Table II are the averages of the percentage deviations, summed without respect to sign, of the weights predicted by the formulas from the means of the organ weights given in Table I. In considering the significance of the experimental

results we are to present we shall be concerned with gross devia-
tions from the predicted values, and will discuss only those that lie
beyond the range of the greatest + to the greatest − deviation of
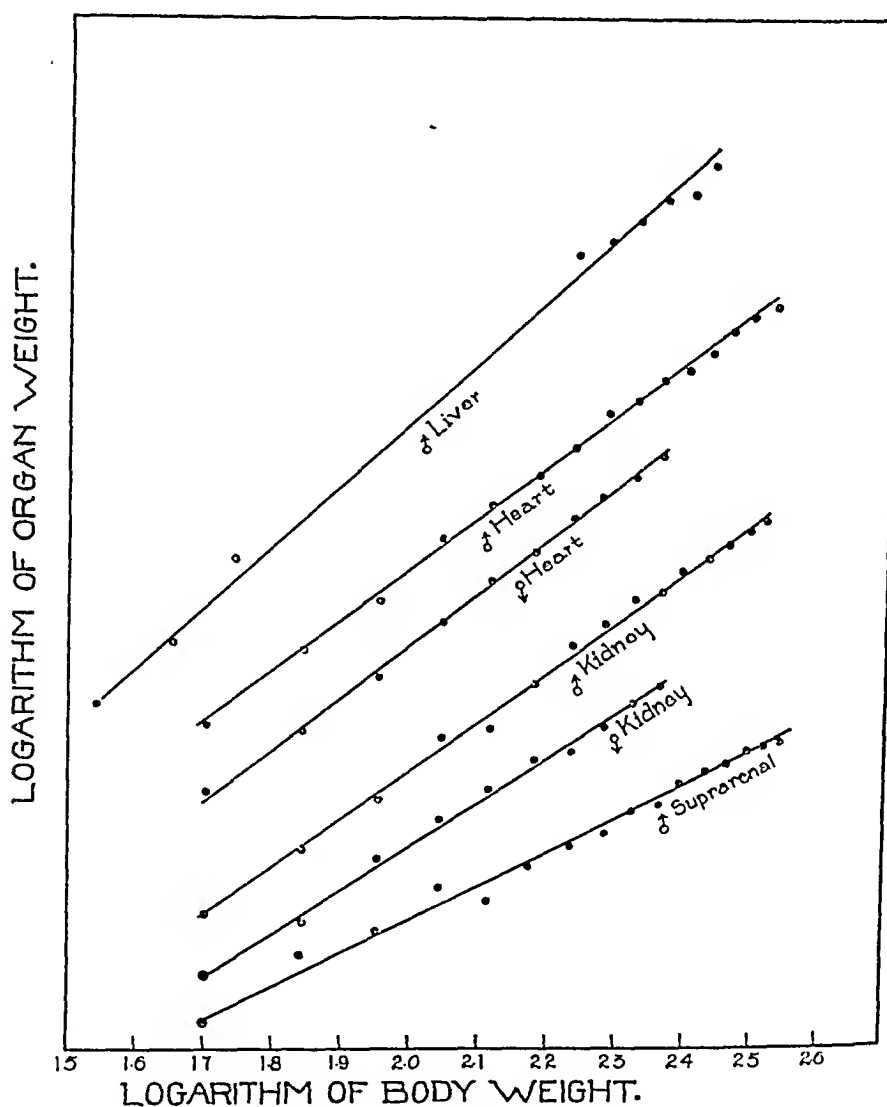
Fig. 1

any of the groups in Table I. These ranges of error are given for
each formula in Table II.

The experimental results in Table III are derived from observations
on groups of rats comparable with those whose organ weights are

given in Table I.   They were subjected to essentially the same condi-
tions[1] with the exception of one experimental variable.   These vari-
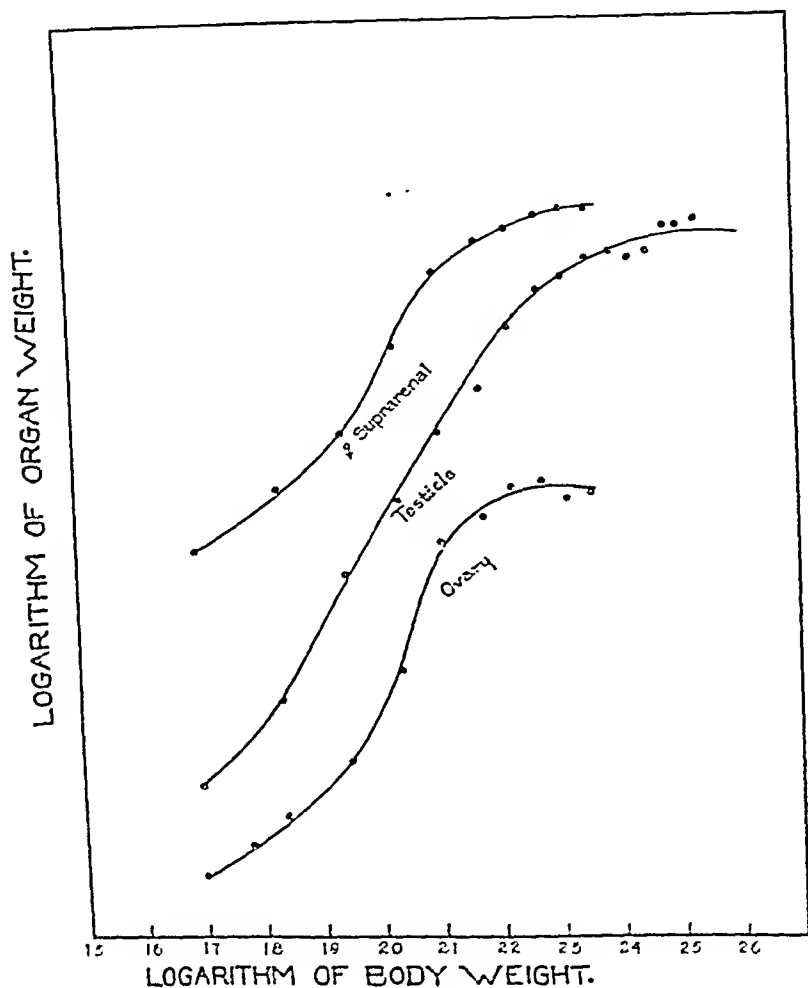
FIG. 2

[1] The housing and temperature conditions were identical and the diet was the
same.   Except in the thyroidectomy group there was no operation.   In the preg-
nancy experiment a diet that contained 16 per cent of casein with corn starch,
lard, vitamin, and mineral supplements was used.   In other experiments this
dietetic change had led to no appreciable deviation from the organ weights found
on the usual diet.

ables are classified as dietetic (change in protein consumption), anabolic increase (pregnancy), metabolic decrease (thyroidectomy), and metabolic increase (thyroxin administration). Those instances in which the organ weights deviated from the predicted values beyond the ranges of error given in Table II are printed in bold-faced type.

Although we are well aware that the simplicity of these experiments is only apparent and that a great multiplicity of factors, many of them unknown, are in reality involved, and although we have had experience with the possibilities of error in dealing with weight measurements of such a changing mixture of materials as are combined in the body and its organs, nevertheless we advance the opinion that the all important factor that determined the organ weight

TABLE II

Formula	Average prediction error	Range of error
	per cent	*per cent*
♂ H.W. $= 12.6$ (B.W.)$^{0.750} + 8.0$	1.6	$+3.0$ to -2.6
♀ H.W. $= 12.6$ (B.W.)$^{0.750} + 1.5$	0.7	$+1.8$ to -1.0
♂ K.W. $= 30.0$ (B.W.)$^{0.717} + 20.6$	1.7	$+4.6$ to -3.2
♀ K.W. $= 40.4$ (B.W.)$^{0.648} + 12.2$	1.7	$+5.1$ to -1.6
♂ L.W. $= 92.0$ (B.W.)$^{0.838} - 96.0$	3.7	$+7.0$ to -7.0

H.W., K.W., and L.W. are respectively the weights of the heart, both kidneys, and liver in milligrams, while B.W. is the body weight in grams.

changes given in Table III was the amount of work imposed on these three organs. In support of this hypothesis the following considerations are advanced.

The work of the heart has been calculated from the rate of volume flow of blood and the mean arterial pressure in heart-lung preparations. Although we have no such measurements in our experiments we know in general that any factor that changes the rate of volume flow of blood will change the amount of work done by the heart so that in deciding whether any of our experimental variables increase or decrease heart work we may use what is generally accepted with respect to the effect of these variables on the rate of blood flow. Now it is shown in Table III, that, in contradistinction to the pro-

nounced effects on the kidneys and liver, changes in protein consumption leave the heart weight unchanged, although the decreased metabolism of protein starvation and the increased metabolism due to the specific dynamic effect of protein should have been accompanied by

TABLE III

Experimental variable	Conditions	Number of rats	Age	Sex	Body weight	Observed weights				Relation to predicted weight			
						Heart	Kidneys	Liver	Protein of liver	Heart	Kidneys	Liver	Protein of liver
			days		gm.	mg.	mg.	mg.	mg.	per cent	per cent	per cent	per cent
Diet changes	No protein but otherwise adequate diet for 10 days	90	110	♂	195	676	1199	6874	1220	+1	−10	−8	−16
	No food for 7 days	90	110	♂	186	657	1163	4900	1042	+2	−10	−32	−25
	Casein in large amounts for 7 days	30	110	♂	194	663	1630	7573	1632	±0	+23	+1	+16
	Liver proteins in large amounts for 7 days	30	110	♂	222	725	1715	11495	2306	−1	+17	+37	+42
Anabolic increase	Pregnancy. Observed 2 days before term	20	100	♀	232	623	1114	8835	1602	−17	−20	+1	+5
Metabolic decrease	Thyroidectomy. Observed 31 days after operation	41	140	♂	192	554	1040	6280	1251	−16	−21	−16	−12
Metabolic increase	Thyroxin administered for 9 days	30	110	♂	210	1010	1845	8376	1725	+44	+30	+5	+15

corresponding changes in the volume flow of blood from the heart which, under the terms of our hypothesis, should have altered its weight. But it must be remembered that the metabolic effects of protein have been determined under basal conditions that did not exist in our experiments. Dock and Lewis (4) have shown that in rats heat production is determined mainly by heat loss and that factors which at an environmental temperature of 28°C. and under basal conditions have pronounced metabolic effects, at a temperature

of 20°C. and under ordinary conditions have their effects largely nullified by compensating changes in the heat production of the voluntary muscles. This damping of the degree of metabolic effect under our special conditions must also be taken into account in interpreting the effect of thyroidectomy and thyroxin administration. Thus it is known that thyroidectomy induces a decrease of about 40 per cent in basal metabolism and so presumably in rate of blood flow, and yet in our experiment there is a decrease of only 16 per cent in heart weight. The conditions in our experiments are so complex that we cannot expect any quantitative parallelism between our heart weights and metabolic measurements made under other and simpler conditions, and we must be content with observing the direction rather than the degree of change. Certainly in the dietetic experiments there was an obvious difference in the activity of the groups deprived of protein and of those given a large surplus of protein, for the former were constantly searching for the food they needed while the latter were quiet and replete, and this difference is one that would diminish the metabolic effects of the variation in protein consumption. But, in addition to this factor, there is another and more general consideration to be taken into account. In the experiments in which no protein was given, the animals, at the time they were killed, had less fat than the controls. Since the heart of the rat has little fat and the body contains a good deal, there was a proportionally greater loss of weight of fat from the body than the heart, so that $H.W./B.W.^{0.750}$ which in essence is our predicted heart weight, would under these conditions be increased. It is this circumstance, more than any other, that may lead to error when we try to compare experimental and control observations on rats of different body weights and different degrees of fatness by the device of expressing the two sets of organ weights in terms of weight per unit of body weight, body surface, or, as here, in terms of some empirically determined power of the body weight. Such quantitative comparisons are valid only if the proportion of fat and water in the bodies of the two groups are alike. Yet for such qualitative purposes as concern us here, where we are considering the significance of gross increases or decreases of organ weight, this quantitative uncertainty need not debar us from drawing

efinite conclusions. Thus, for instance, the 10 per cent decrease in idney weight in fasted rats is not to be put aside as meaningless, for 'e know that the loss of body fat is a factor that, in itself, will induce n increase and not a decrease in the relation between the actual and redicted weights.

The remaining heart weight change shown in Table III, the 17 er cent decrease of heart weight in pregnancy, is a particular example f the general fallacy involved when formulas from animals under ne set of conditions are used to compare organ weights from ani- nals in which other conditions have induced a change in the propor- ions of the components which make up the body weight. The aver- age body weight of these pregnant rats was 232 gm. and under our standard conditions the heart predicted by the formula is 749 mg. or 17 per cent more than the 623 mg. of heart actually found. But 27 gm. of this 232 gm. of total body weight consisted of embryos which had hearts doing the physical work of circulating blood throughout their bodies. If on this account, neglecting the work of supplying oxygen to the embryos, we subtract their body weight, we leave a net maternal body weight of 205 gm. This gives a pre- dicted heart weight of 682 mg. which is still 8 per cent more than the actual. But it happens that in this case we have protein deter- minations on the heart and bodies of the pregnant and of a special control group of non-pregnant rats, and though there is not much difference in the concentration of protein in the hearts, we find that the 205 gm. of maternal body has only 14.26 per cent of protein while the controls had 15.86 per cent. The protein in the bodies of the pregnant rats was thus diluted with over 9 per cent of some ma- terial not present in the controls. There was no obvious difference in fat content and in this case the added material was water, a sub- stance that takes no oxygen from the blood and that, apart from the additional energy required to move a heavier body weight, leads to no increase in cardiac output. When this weight of water is sub- tracted from the body weight or when the comparison is made on the basis of the protein content of the heart and body there is no longer any difference between the control and pregnant rats.

The work of the kidney is osmotic work and is the energy used in concentrating the glomerular filtrate. It varies directly as the sum of

the rates of excretion of each urinary constituent multiplied by the $\log_n$ of the ratio between the concentration of each urinary constituent in the urine and plasma (5). In measurements of work done we have found that under conditions similar to those we employed the work of the kidney rises in fairly close correspondence with increase in total nitrogen excretion so that in considering which experiments involve change of work for the kidney we may in general be guided by what is known with respect to their effect on the rate of nitrogen excretion. The decrease in kidney weight when no protein is eaten and the increase when more than the usual amount is consumed follow the known changes in nitrogen excretion. Similarly the decrease in kidney weight after removal of the thyroid gland and the increase when thyroxin is given are in the direction of the changes in nitrogen excretion that occur as a result of changes in protein catabolism and in protein consumption. The 20 per cent decrease in kidney weight in pregnancy is, of course, in part determined by the increased water content of the body. In this case the weight of the embryos must be included since their kidneys were not working and the products of embryonic catabolism were excreted by the maternal kidneys. The protein concentration in the whole 232 gm. of body weight in the pregnant rats was 13.5 per cent as compared with 15.9 per cent in the whole body of the controls. If we assume that all of this dilution was due to water and subtract its weight we have a corrected body weight of 197 gm. and from this a predicted kidney weight of 1250 mg. The actual kidney weight is 1114 mg. and there is still an 11 per cent decrease. This, we believe, is due to a diminution in work on account of the greater utilization of the relatively small amount of protein in the food for the rapid synthesis of protein in the uterus and its contents. This view is supported by the fact that when pregnant rats are given a diet that contains 43 per cent instead of 16 per cent of protein there is an increase instead of a decrease in kidney weight.

In the end the questions we have raised will be answered when experiments can be devised in which the relation between the effective mass of the heart and kidney and the work done by these organs can be measured. In the meantime the considerations we have ad-

vanced are presented, not for their own sake, but as a basis for a study of change in the size of the liver, a field in which there is a need for a preliminary working hypothesis. For while we have a clear idea as to what constitutes the principal work of the heart and kidneys we have no analogous conception with respect to the liver. It may be that the liver has so many functions that no one experimental variable changing only one sort of work will appreciably alter liver size. But we can at least measure the size of the liver under these same conditions and note any agreement or discrepancy between the behavior of liver and heart and kidneys.

There is one difficulty that must be faced before we can approach this problem and that is the circumstance that under certain conditions a not inconsiderable part of the total liver weight consists of fat and glycogen, relatively passive stores of food reserves. We need the actual working machinery stripped of all its fuel and accessories. We believe that the best approach to this is the determination of the total protein content of the liver (6). Part of this liver protein it is true may be a storage protein that comes and goes in accordance with the supply and demand of protein for metabolism. There is, however, accumulating evidence that this labile liver protein is no specialized, inert, and localized deposit analogous to glycogen (7) but that when it exists it is present as a more or less uniform increase in all the principal protein components of the organ (8).

In Table III we have given both the total liver weight and the weight of liver protein, but only the protein changes are considered since we believe that they alone have any precise meaning with respect to the relation between the work and the functioning structure of the liver. The protein deviations represent the changes from the protein content of the liver of a group of 90 rats kept under the same conditions as those observed in the control animals from which the formulas were derived.

The average protein content of the livers of this control group of 90 rats was 1713 mg. The average body weight was 237 gm. It was assumed that if a curve of liver protein for the whole range of body weights had been measured under the same dietary and other conditions it would have paralleled the line in Fig. 1 for total liver weight and would thus have had the same slope, varying as

the 0.838th power of the body weight. On this assumption the liver protein content of control groups of rats of the same body weight as the experimental groups was calculated by multiplying 1713 mg. by $\dfrac{(\text{experimental body weight})^{0.838}}{(\text{control body weight})^{0.838}}$.

Thus in the first experiment in Table III, the control liver protein content at the experimental body weight of 195 gm. is 1713 mg. $\times \dfrac{195^{0.838}}{237^{0.838}} = 1456$ mg.

In general it will be noted that in the dietetic experiments the direction of change in liver protein is in agreement with kidney weight changes (though there is one remarkable quantitative peculiarity to which we shall return), that in the pregnancy experiment the sign of the deviation is the opposite of that for the heart and kidney, and that in the metabolic experiments all three organs alter in agreement with one another. It is, however, only in the case of the change in liver protein with change in protein consumption that we can go beyond analogy and comparison. The observation made by Dock (9) that it is the liver that uses the greater part of the additional oxygen consumed as a result of the specific dynamic effect of protein is direct evidence in support of the view that work is being done by the liver during the period when amino acids are being absorbed from the portal blood stream.

In the metabolic experiments the results are unambiguous only for the heart because it is the only organ that is not appreciably influenced by changes in food consumption. The removal of the thyroid gland is followed by a decline in the amount of food eaten and the administration of thyroxin leads to an increase of food intake, and so the effect on liver protein might be a secondary result of these associated food changes. We felt it was necessary to clear up this uncertainty with respect to the kidney and liver by repeating these experiments on groups that had fasted for 7 days, and in the case of the liver these experiments should be the more decisive because after a 7 day fast it is to be anticipated that all stores of protein in the liver will have been utilized. But under these new conditions the formulas we have given are no longer applicable. We therefore carried through special control observations, using the same number of rats and contriving the selection in such a way as to get at the end of the 7 days fasting average body weights identical with those of the

experimental groups. However, in the group given thyroxin the loss of body weight was so pronounced that it was not possible to get a comparable control group of the same body weight and in this case it was necessary to calculate the control values from the data given in Table III for 7 day fasted rats.

The body weight of the fasted controls was 186, while the body weight of the fasted thyroxin group was 158 gm. The assumption was made that if continuous curves of organ weight for fasted rats had been obtained against body weight they

TABLE IV

The Effect of Variation in Metabolic Rate after a 7 Day Fast

Experimental variable	Conditions	Number of rats	Age	Sex	Body weight	Observed weights				Deviation from fasted controls of same body weight			
						Heart	Kidneys	Liver	Protein of liver	Heart	Kidneys	Liver	Protein of liver
			days		*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
Metabolic decrease	Thyroidectomy. Observed 29 days after operation	25	110	♂	173	532	969	4462	954	−15	−15	−2	−2
Metabolic increase	Thyroxin administered for 9 days	20	110	♂	158	827	1342	4995	1055	+40	+30	+17	+15
	Dinitrophenol administered for 7 days	30	110	♂	183	641	1155	4676	1018	±0	−3	−7	−6

would have paralleled the lines in Fig. 1 and would have had the same slopes. On this assumption the control heart for the thyroxin group is $657 \times \frac{158^{0.75}}{186^{0.75}} = 592$, the control kidneys $1163 \times \frac{158^{0.717}}{186^{0.717}} = 1034$, the control liver $4900 \times \frac{158^{0.525}}{186^{0.525}} = 4280$ and the control liver protein $1042 \times \frac{158^{0.525}}{186^{0.525}} = 910$.

The results given in Table IV indicate that under these conditions thyroidectomy leads to no significant decrease in liver protein relatively to fasted controls. On the other hand, thyroxin, even in fasted rats, leads to a 16 per cent increase in liver protein. The heart

changes are almost the same as those in fed rats and in the kidney only the thyroidectomy decrease is lessened.

This demonstration of the pronounced effect in both fed and fasted animals on heart, kidneys, and liver protein of an increase in metabolic rate induced by thyroxin, raises the question as to whether all of the organ weight changes we have found may be due to alterations in the rate of metabolism of the organs themselves. Under this view, for instance, the alteration of kidney and liver protein with change in protein intake would be ascribed not to the need of more oxygen for work alone but to the fact that in these organs, the metabolic rate of the whole kidney and liver, not only of its directly working parts, varied with protein intake. This hypothesis can be tested by observing the effect of dinitrophenol which markedly increases organ metabolism without increasing the work of the heart, since the additional oxygen required is obtained not by increased output of blood by the heart but by taking more than the usual proportion of oxygen from the blood in the capillaries and without increasing the work of the kidneys since under its influence there need be no increase in protein catabolism. The results as shown in Table IV show no increase in heart, kidney, or liver protein after dinitrophenol in spite of the fact that in the doses used there was a pronounced increase in organ metabolism (10). We conclude therefore that the mechanism responsible for change in organ size is not the total metabolic rate within the organ, and revert to the hypothesis that it is work that determines organ size though it may well be that the special oxygen requirements entailed by work are an essential part of the mechanism by which the equilibrium between work and organ size is maintained.

The general agreement between the direction and extent of the kidney and liver changes suggest that whatever increases the work of the kidney generally increases also the work of the liver. But there are two rather marked discrepancies in Table III.

In the pregnancy experiment the heart and kidneys became much smaller relatively to the body weight but this is not true of the liver protein. Instead of a decrease a 5 per cent increase was found. This is the more remarkable because the two considerations which led us to believe that the decrease in heart weight was only apparent (the addition of water to the mother's body and the work of the fetal

hearts), both apply in the case of the liver. If similar corrections of maternal body weight are applied here we find that the liver is not 5 per cent but 21 per cent greater than the controls. This result suggests that in pregnancy the liver may have some special work to do that leads to its enlargement.

The other disagreement does not involve any change of direction and is only quantitative, but the 42 per cent increase in liver protein when large amounts of dried liver are taken as food is of a different order of magnitude in relation to the other liver changes. A similar experiment with dried whole kidney as food gave almost as great an increase and so we can conclude that this result is not due to any substance peculiar to liver tissue. Since an equally large consumption of casein, which contains no nucleic acid, led only to a 16 per cent increase in liver protein, the first possibility to explore would be that the metabolism of nucleic acid imposes a special load of work on the liver. Experiments to decide this point are now under way.

In conclusion it is well to remember that what we have called in this paper experimental variables are not changes of only one factor at a time within a system in which all the important variables are known and measured. Even when the only variation consisted in giving more or less protein as food, or in adding or subtracting thyroxin, much more was effected than an alteration of protein supply or rate of metabolism. The weights of the heart, kidneys, and liver protein were doubtless influenced by a multiplicity of factors other than those we have singled out and among them may be many whose operation we do not even suspect. So our opinion that the observed facts are at present best accounted for by the supposition that the main factor that determines the weight of the heart, kidneys, and liver protein is the amount of work these organs are required to perform is only a working hypothesis. It is presented because it may be useful in planning further experiments with other and more precise methods for the analysis of the mechanism of change in the amount of functioning liver tissue and in the investigation as to what constitutes its principal work.

CONCLUSIONS

1. The ratios between the rates of growth of the body and of the heart, kidneys, and liver are approximately uniform between 40 gm. body weight and the body weight at maturity in the albino rat.   The male and female hearts grow at 0.75 times the rate of growth of the body, the male kidneys at 0.717 times, the female kidneys at 0.648 times, and the liver at 0.838 times the rate of growth of the body as a whole.

2. Formulas for the prediction of organ weight from body weight were derived from the data on 1591 albino rats kept under constant conditions.

3. A series of experiments in which dietetic and metabolic variables were introduced into otherwise constant conditions showed that the heart weight was not affected by diet, and that both kidney weight and weight of liver protein (used as a measure of effective liver size) varied in the direction of change in the protein content of the diet.   Decrease in rate of metabolism induced by thyroidectomy and increase in metabolism following the administration of thyroxin led to a corresponding fall and rise of heart, kidney, and liver protein weight.   These results were confirmed in experiments on fasted rats with the exception that under these conditions thyroidectomy did not appreciably decrease liver protein weight relatively to fasted controls. Increase in organ metabolism due to dinitrophenol had no effect on organ weight.

4. When experimental changes alter the composition of the body with respect to fat or water, the comparison of experimental and control organ weights in terms of any one function of body weight is fallacious.

5. Conditions that change kidney weight usually change liver protein weight in the same direction and roughly to the same degree. The possible meaning of two exceptions to this rule is discussed.

6. The observations made are regarded as supporting the hypothesis that, after weaning, change in the weight of the heart, kidney, and liver protein is determined mainly by change in the amount of work done by these organs.

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NEPHRITIS AND ITS INFLUENCE UPON HEMOGLOBIN PRODUCTION IN EXPERIMENTAL ANEMIA

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It is common knowledge that in human cases of glomerulonephritis a persistent anemia is frequently observed. In this connection there is legitimate interest in cases of spontaneous progressive glomerulonephritis in dogs which were included in the anemia colony. These dogs were under observation from birth to death and the hemoglobin production studied continuously through several years to death in uremia. A glance at the summary table below shows that even during the last year of life these dogs retain about two-thirds normal hemoglobin production. It is very doubtful whether this degree of incapacity would result in *spontaneous anemia* in such dogs. During the last month or two when the non-protein nitrogen is high and the dog on the verge of uremia, the hemoglobin production may fall to zero in some cases (Table 6) but again may be only slightly impaired (Tables 1 and 3).

In contrast to this picture where the *nephritic* dog shows but little incapacity to produce hemoglobin under standard conditions we refer to similar experiments (2) to show that *infection* causes serious or even complete incapacity to produce hemoglobin. Experiments are reported to give convincing evidence that endometritis or sterile abscesses will cause almost complete inhibition of hemoglobin production in the standard anemic dog. One may raise the question therefore whether in man the renal injury rather than some obscure focus of inflammation may be largely responsible for the anemia associated with chronic nephritis.

Glomerulonephritis in the anemia colony is not rare and 11 per cent of the dogs show it at autopsy. We note frequently endocarditis which may have been a factor. As one cause of the endocarditis or

nephritis or both we suspect distemper.   Distemper was common in the colony in the early years of this program but has been largely eliminated by the use of the Laidlaw-Dunkin vaccine.   Intestinal infections with paratyphoid or dysentery organisms occur rarely in these dogs but this may be a factor.

Glomerulonephritis in dogs as we observe it begins insidiously with albumin, casts, and a few red cells in the urine.   There may be no clinical evidence of nephritis for years but we may observe short periods of low food consumption with or without vomiting.   Gradually over one or several years the non-protein nitrogen of the blood increases, the casts become more numerous and functional tests show decreased renal elimination.   Acute nephritis may supervene at the end.   Food consumption fortunately is usually excellent even up to the last week or so preceding uremia.

Anatomically the kidneys present the general picture observed in human glomerulonephritis.   The kidneys show a scarred surface, the cortical striations are obscured and the cortex often narrowed.   Histologically the glomeruli show adhesions between the capsule and glomerular tuft.   The glomeruli may be large or small and may show hyaline degeneration.   Casts are numerous in the tubules which show abnormal epithelium.   Nests of mononuclears appear frequently in the stroma of the cortex and the stroma is increased in amount. Acute nephritis is often superposed.

Methods

Dogs in the anemia colony are kept under uniform conditions.   They are born and raised in the animal house, protected against distemper and other infections, yet many of them develop a true nephritis.   The routine relating to these dogs has been described in detail (3).   Although not given in the condensed tables (except Table 3) the dog is bled each week to maintain a constant anemia level or stimulus for hemoglobin production.   Complete red counts, red cell hematocrit, hemoglobin determination, and blood volume measurements are done each week. The urine is examined for casts, albumin, and red cells at intervals depending upon the clinical condition.   Non-protein nitrogen of the blood and renal function tests are done and recorded in the clinical histories of each dog.   All dogs listed in these experiments died with high non-protein nitrogen figures and the general picture of uremia.

The liver extract with and without iron has been described elsewhere (4).   It is potent in anemia due to blood loss as indicated in Table 7 and elsewhere.   It

does not contain the principle potent in pernicious anemia. The pernicious anemia extract (343) contains only 10 per cent of the potent factor active in anemia due to blood loss.

EXPERIMENTAL OBSERVATIONS

If the information about the progress of the nephritis is meagre the reader should remember that the primary objective of our studies is the physiology of hemoglobin production and this report is a by-product. With a considerable anemia colony to be followed the

TABLE 1

Hemoglobin Production Uninfluenced by Nephrosis, Glomerulonephritis, and Uremia

Dog 24-26.

Year	Pig liver	Pig kidney	Iron 40 mg.	Liver extract — iron	Liver extract + iron	Apricots	Albumin in urine	Blood N.P.N.
	Net hemoglobin produced due to diet factors— *gm. per 2 wks.*							
								mg. per cent
1925	55, 39							
1926	*75						+	
1927	46, 73							
1928			55					
1929			42	31	60			
1930					62		+	
1931			42	51	41	44		
1932	80	70	41	50			+	57
1933		49			†61		+	244
								277

* Chicken liver (equivalent to pig liver).

† Death 1½ weeks after completion of this experiment.

amount of work which can be done by the staff is finite and must be carefully allocated. The fact remains that all these dogs included in this report developed spontaneous nephritis and died as a result of the nephritis without conspicuous and uniform depression of hemoglobin production.

Clinical Experimental History and Autopsy.—Table 1. Dog 24-26. Adult male bull mongrel, weight 10 kilos. Born 1923, 10 years old at death. Uneventful anemia history 1925–26.

Apr., 1926. First urine analysis: Albumin, granular casts, few red cells. Other than occasional poor food consumption, no clinical symptoms. Weight 10.5 kilos. *Feb., 1928.* Extreme dental caries and pyorrhea. Treated with improvement. Weight 10.3 kilos. *Sept., 1929.* Teeth again caused trouble. Several removed. Uneventful anemia history. Weight 10.0 kilos. *July 5, 1930.* Following blood removal several convulsions, salt intravenously. Recovery. *Sept., 1930.* Urinary findings: Albumin, casts, and few red cells. Weight 10.1 kilos. *Oct., 1930.* Pyorrhea as before. Marked salivation. Gradual improvement on meat diet. *Nov., 1930.* Left food for several days. *Dec., 1930.* Urine findings unchanged. Several teeth removed. *Feb., 1931.* Gradual loss of weight, stabilized at 9 to 10 kilos. (Weight from beginning uniform 10 to 11 kilos.) *Apr., 1932.* Left considerable food. Weight 10.0 kilos.

May 4, 1932. Non-protein nitrogen 57 mg. per cent. Urea clearance 20 per cent normal.

May, 1933. Urine findings unchanged. Weight 9.2 kilos. History uneventful to July, 1933.

July 8, 1933.—Liver feeding experiment not completed. Food consumption 80 to 36 per cent. *July 11.* Sick, salivated, left all food. Weight 9.1 kilos. Sugar intravenously. Non-protein nitrogen 244 mg. per cent. *July 12.* No improvement. Non-protein nitrogen 277 mg. per cent.

July 12, 1933. Killed with ether. *Autopsy* done at once. Heart is enlarged. There are no acute vegetations but the mitral valve shows a little scarring and old healed endocarditis. Lungs and other viscera normal. Kidneys are small. Surface is scarred and pale. Cortex on section shows scars and obliterated striation. Histological sections show arteriosclerosis and nephrosis but much acute and chronic nephritis in addition. Many glomeruli show adhesions to the capsules and others are normal. Scars are numerous and contain nests of mononuclears. Casts are not numerous but many tubules show an acute exudate of polymorphonuclears. Many small cysts are present.

Table 1 (dog 24-26) shows a continuous history of this anemic dog lasting 8 years. Albumin was first noted in the urine in 1926. The dog had dental caries and severe pyorrhea necessitating removal of several teeth, 1926–32. It was suspected that the mouth infection might be responsible for some of the low values for hemoglobin production (pig liver). In 1932 the renal function (urea clearance) was about one-fifth normal and the non-protein nitrogen of the blood remained elevated. There was rapid progress of the nephritis toward uremia in 1933 with very high figures for non-protein nitrogen of blood (244 to 277 mg. per cent).

This dog with a severe progressive nephritis in 1933 was able to

produce a low yet normal amount of new hemoglobin on various standard diets.

Clinical Experimental History and Autopsy.—Tables 2 and 3. Dog 26-164. Adult male coach bull mongrel, weight 12.2 kilos. Born 1925, 12 years old at death. Anemia history 1930-37. Uneventful to Feb., 1934. Routine anemia experiments.

Sept., 1930. Urine: Normal findings. Occasional period of poor food consumption. Weight 12.5 kilos. *1933.* Potato diet for 2 months reduced plasma protein to low normal of 5.4 per cent.

TABLE 2

Hemoglobin Production Depressed at Times with Glomerulonephritis and Uremia Dog 26-164.

Year	Pig liver	Pig kidney	Iron 40 mg.	Iron 400 mg.	Liver extract − iron	Liver extract + iron	Albumin in urine	Blood N.P.N.
	Net hemoglobin produced due to diet factors—*gm. per 2 wks.*							
								mg. per cent
1930			35				0	
1931	82				50, 60 49			
1932					38, 68			
1933		57	54					
1934	92, 89	43				68	++	33
1935	51	48	49	79	0	59, 69	++	170 63
1936		17	8, 39			44	+	71
1937		*15					++	230

* Death 5 weeks after completion of this experiment.

Feb., 1934. First evidence of nephritis. Urine showed considerable albumin, granular casts, red cells. Similar findings June and September. Other than occasional poor food consumption dog showed no clinical symptoms. Weight 12.3 kilos. *Mar. 1, 1934.* Non-protein nitrogen 33 mg. per cent. *Mar. 20, 1935.* Following ferrous sulfate experiment (Fe 400, Table 3) during 5th and 6th weeks "bread after period," dog left 65 to 75 per cent food. Weight 12.8 kilos. Glucose 3 doses daily, 3 days. *Mar. 20.* Non-protein nitrogen 99 mg. per cent. *Mar. 21.* Non-protein nitrogen 170 mg. per cent. Plasma proteins 6.8 per cent. *Mar. 22.* Non-protein nitrogen 63 mg. per cent. Food consumption 75 per cent. Glucose intravenously 2 doses daily. *Mar. 24.* Non-protein nitrogen 70 mg. per cent. *Mar. 23-27.* Sugar intravenously 2 doses daily. Food consumption averaged 80 per cent. *Mar. 29.* Non-protein nitrogen 53 mg. per cent.

Sugar intravenously 1 dose, 6 days. *Apr. 4.* Non-protein nitrogen 47 mg. per cent, food consumption 100 per cent. *Apr. 11.* Non-protein nitrogen 50 mg. per cent.

May 2. Non-protein nitrogen 28 mg. per cent. Urine showed few casts only, occasional red cell. Uneventful history to Oct., 1935. Food consumption 90 to 100 per cent (urine protein excretion 1 gm. 24 hrs.). Weight 13.0 kilos. Slight vomiting occasionally. Drinks much water. Leaves food at times. *Nov., 1935, to Jan. 29, 1936.* Soy bean meal partially replaces bread, consumption 90 to 100 per cent. *Jan. 22, 1936.* Urine: Albumin, no casts, occasional red cell. Weight 12.8 kilos. *Mar., 1936.* Soy bean diet: Soy bean mush 500 gm., salmon bread 100 gm. daily. *May, 1936.* Urine findings as before. Anemia history uneventful to July. Weight 10.4 kilos. *July 27.* Left food 2 days. *July 31.* Non-protein nitrogen 71 mg. per cent. Dog quiet but does not act sick. *Sept. 22.* Plasma proteins 6.3 per cent. *Jan. 5, 1937.* Plasma proteins 6.4 per cent. *Jan. 6.* 24 hour urine protein excretion 0.1 gm.

Jan. 13. Urine: Albumin trace, no casts, occasional red cell. Weight 10.6 kilos. Again excessive water drinking. No particular clinical symptoms to Feb. 8. Food consumption 100 per cent. *Jan. 27.* Salmon bread basal diet. *Feb. 8.* Left all food. Seems sick. Temperature 37.0°, leucocytes 22,800. Weight 9.9 kilos. Non-protein nitrogen 230 mg. per cent. Sugar intravenously 3 doses.

Feb. 9, 1937. Found dead in morning, body cold. *Autopsy* done at once. Heart shows hypertrophy of left ventricle and acute mural vegetations in left auricle above the mitral valve (cultures of hemolytic *Staphylococcus aureus*). Lungs show some bronchopneumonia and edema. Kidneys show a scarred surface, narrow and scarred cortex on section, scattered ecchymoses in parenchyma. Histological sections show an advanced acute and chronic glomerulonephritis. Stroma is much increased and contains great numbers of mononuclears in clusters. Some tubules contain polymorphonuclears and many casts are seen.

Table 2 (dog 26-164) shows 7 years of continuous history of this anemic dog. Nephritis was not recognized until 1934. During March, 1935, the dog was close to uremia (see Table 3) but made a good recovery, lived to 1937 and died in uremia. During 1935–37 we note a few periods in which the hemoglobin production on standard diets (pig kidney and iron) was definitely below normal but this was not a constant finding. The blood non-protein nitrogen was above normal on many occasions during a 2-year period 1935–37, Table 2. There was terminal endocarditis and bronchopneumonia.

Table 3 (dog 26-164, see history above) shows a period of 15 weeks in which the dog came close to uremia but made a good recovery. Table 3 gives the usual experimental data (except red cell count and

hematocrit) which show that the anemic dog produced a net amount of hemoglobin (79 gm.) due to iron feeding which is normal just *before the uremic period* and a low normal hemoglobin (69 gm.) output due to liver extract plus iron just after the uremic period while

TABLE 3

Hemoglobin Production—Dog Close to Uremia with Recovery
Dog 26-164.   March, 1935 (see Table 2 and History).

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	Blood Hb. level	Blood Hb. removed	Blood N.P.N.
Food, gm. per day	per cent	kg.	cc.	per cent	gm.	mg. per cent
Fe 400 mg., bread 350, salmon 100, Klim 20	100	12.9	734	60	40.4	
Fe 400 mg., bread 350, salmon 100, Klim 20	100	12.7	756	59	34.3	
Bread 350, salmon 100, Klim 20	100	12.9	768	47	20.6	
Bread 350, salmon 100, Klim 20	91	13.1	779	45	1.2	
Bread 350, salmon 100, Klim 20	85	13.2	800	45	12.8	
Bread 350, salmon 100, Klim 20	91	13.1	803	45	11.3	
Bread 325, salmon 100, Klim 20	75	12.8	810	41	1.2	

Severe Nephritis (Uremia) and Recovery.   N.P.N. 99—170—63 mg. per cent
Mar. 20-24

Bread 300, salmon 200, Klim 20	79	13.4	785	42	1.1	70
Liver extract + Fe 300 mg.*	90	12.9	770	68	12.7	53
Liver extract + Fe 300 mg.*	100	12.9	707	57	20.3	47
Bread 250, salmon 150, Klim 20	100	13.2	741	55	24.3	50
Bread 250, salmon 150, Klim 20	100	13.2	788	50	20.4	—
Bread 275, salmon 150, Klim 20	100	13.2	800	47	1.3	—
Bread 275, salmon 150, Klim 20	86	13.3	803	46	10.7	28
Bread 250, salmon 150, Klim 20	91	12.9	767	40	1.1	—

* Bread 200, salmon 150, Klim 20 daily.

the blood non-protein nitrogen was subsiding to normal.   It is not safe to claim that the hemoglobin producing mechanism in this dog was disturbed by the presence of a severe nephritis.

Clinical Experimental History and Autopsy.—Table 4.   Dog 27-238.   Adult female coach bull mongrel, weight 13.8 kilos.   Born 1928, 9 years old at death.

Continuous anemia history Nov., 1928, to Aug., 1937. Raised on bread and milk diet from weaning to adult age; dog never received any animal viscera or meat during entire life.

Sept., 1930. Urine: Albumin trace, no casts, 2 to 5 red cells per field, following iron (Fe) intravenous experiment. No abnormal clinical symptoms. Weight 15.4 kilos.

Uneventful anemia history to *Sept., 1931,* dog begins to leave food at times. Weight 16.0 kilos. *July 14 to Aug. 14, 1933.* Plasma protein 5.6 to 6.5 per cent. Weight 16.2 kilos. *Oct. 21, 1933.* Urine: Albumin trace, no casts, few red cells. *Sept. 22, 1934.* Urine: Albumin +, no casts, no red cells. Weight 16.2 kilos.

TABLE 4

Hemoglobin Production Shows Terminal Depression with Glomerulonephritis and Uremia

Dog 27-238.

Year	Iron 21 mg.	Iron 40 mg.	Iron 400 mg.	Apricots	Albumin in urine	Blood N.P.N.
	Net hemoglobin produced due to diet factors—*gm. per 2 wks.*					
						mg. per cent
1929	40					
1930		47, 42	79	0	+	
1931	29	60				
1932		49	65			
1933				33	+	
1934		53			+	
1935		44, 63	65, 66			
1936		59			++	
1937		*7			++	200

* Death 4 weeks after completion of this experiment.

Sept., 1935. Weight 16.6 kilos.

Uneventful anemia history to May 6, 1936.

May 6, 1936. Urine: Albumin ++, no casts, 3 to 5 red cells per field. Weight 16.0 kilos. Food consumption good to Aug. 16, 1937. *Aug. 16, 1937.* Leaves food 2 days. Slight vomiting. Weight 13.3 kilos. *Aug. 19.* Dog seems sick. Sugar intravenously 2 doses. Blood transfusion. Non-protein nitrogen 200 mg. per cent.

Aug. 20, 1937. Found dead in morning, body cold. *Autopsy* done at once. Heart shows hypertrophy but no endocarditis. Lungs show interstitial pneumonia and edema. Kidneys are small, surface granular. Cortex is narrow and shows loss of striation on section. Other organs not related. Histological sections show the usual type of glomerulonephritis with adhesions between glomerular tuft and capsule. The stroma is greatly increased and contains nests of mononuclears. Tubules contain many casts.

Table 4 (dog 27-238) shows more than 8 years of continuous anemia with periodic tests of hemoglobin production which remains close to normal up to the last test completed 4 weeks before death. Renal injury was noted in 1930 but there was no obvious increase in the nephritis until 1936. One negative test with apricots we cannot explain. The other apricot test was slightly subnormal in 1933. The series of tests with iron (40 mg. per day) is very complete and satisfactory. The hemoglobin production with this standard iron intake varies from 42 to 63 gm. which is within normal limits. The hemoglobin production with the same dose of iron just 4 weeks before death shows a definite drop to 7 gm. indicating some impairment of

TABLE 5

Hemoglobin Production Shows No Depression within 3 Weeks of Death Glomerulonephritis and Uremia

Dog 24-49.

Year	Chicken liver	Chicken gizzard	Pig kidney	Beef muscle	Apricots	Albumin in urine	Blood N.P.N.
	Net hemoglobin produced due to diet factors—*gm. per 2 wks.*						
							mg. per cent
1925					52		
1926	83	90					35
1927				25		++	52
1928			55		*48	++	86
							170

* Death 3 weeks after completion of apricot experiment.

hemoglobin production in the last stages of this fatal nephritis. There was a terminal pneumonia.

Clinical Experimental History and Autopsy.—Table 5. Dog 24-49. Adult female bull terrier, weight 13.0 kilos. Born 1923, 5 years old at death. Continuous experimental anemia Oct. 31, 1924, to May 15, 1928. 1924 weight 13.0 kilos. 1925 weight 15.0 kilos. 1926 weight 16.5 kilos. Uneventful anemia history to Feb. 15, 1927.

Feb. 15, 1927. Two convulsions. Otherwise acts like normal dog. Food consumption 100 per cent, bread diet. Weight 15.8 kilos. *Feb. 18.* Vomited food. Temperature 102°. *Feb. 21.* Urine: Albumin, hyaline cast, few red cells. *Feb. 23.* Urine, 24 hours: Albumin 4.5 gm. per liter (Esbach), numerous granular casts, 10 to 25 red cells per field. *Feb. 24.* Blood non-protein nitrogen 35 mg. per cent.

Apr. 1. Ear infection, leucocytes 20,000. Food consumption 100 per cent. Weight 16.1 kilos. Urine: Albumin 4.5 gm. per liter (Esbach), numerous granular casts, moderate number red cells. *Apr. 15.* Leucocytes 17,000 per c.mm. blood. *Apr. 17.* Leaves food intermittently, average consumption for week 75 per cent.

Apr. 26. Urine, 48 hours: Albumin 2.5 gm. per liter (Esbach), granular casts, red cells. Blood non-protein nitrogen 34 mg. per cent. Food consumption for week average 72 per cent. Uneventful history to Sept. 2. *Sept. 2.* Ear discharging again. Leucocytes 19,000 per c.mm. Weight 15.7 kilos.

Dec. 24. Manganese feeding experiment 115 mg. daily. Convulsions after 10 doses. Discontinued. *Jan. 5, 1928.* No clinical improvement. Urine findings same, leucocytes 23,400 per c.mm. Several convulsions. Vomiting. Forced raw liver feeding. *Jan. 14.* Gradual improvement and recovery. Weight 12.2 kilos. *Mar. 15.* Convulsions. Blood non-protein nitrogen 52 mg. per cent. Dog sick, left 50 per cent of food. Albumin 5 gm. per liter (Esbach). *Mar. 19.* Phenolsulfonephthalein test, 2 hours output, 47 per cent. *Mar. 22.* Blood non-protein nitrogen 60 mg. per cent. Food consumption 100 per cent. Weight 15.2 kilos. *Apr. 23–25.* Vomits, leaves food. Blood non-protein nitrogen 86 mg. per cent. Gradual improvement, milk and syrup feeding by stomach tube. *May 25.* Blood non-protein nitrogen 66 mg. per cent, leucocytes 12,400 per c.mm. Food consumption 100 per cent. Weight 14.5 kilos.

June 4–14. Leaves considerable food. Leucocytes 12,600 per c.mm. Infection in ear flares up. *June 15.* Considerable vomiting. Blood non-protein nitrogen 170 mg. per cent. Weight 12.0 kilos.

June 17, 1928. Dog found dead, body warm. *Autopsy* done at once. Heart shows great hypertrophy especially of left ventricle. Both auricles above valves show granules of old and recent vegetations (not unlike *viridans* type). No ulcers in endocardium. Kidneys large and show a granular surface. Architecture of cortex on section is not regular. No hemorrhages. Other organs not related or normal. Histological sections show advanced glomerulonephritis. Almost every glomerulus is enlarged and shows adhesions to the capsule. Tubules show epithelial degeneration and many casts. Stroma shows scars and nests of mononuclears.

Table 5 (dog 24-49) shows an anemia history which runs through 4 years. The determinations of the levels of hemoglobin production are not frequent but have a great interest during the last months of life. Nephritis was observed early in 1927 and was of a rather malignant type probably even at that time associated with a low grade endocarditis. Hemoglobin production was within normal limits whenever tested even late in 1928. The apricot experiments are of particular interest; the first one in 1925 is normal (52 gm. hemoglobin) and the second one late in 1928 completed only 3 weeks before

death is likewise normal (48 gm. hemoglobin) in spite of impending
uremia.

Clinical Experimental History and Autopsy.—Table 6. Dog 27-233. Adult
male coach terrier mongrel, weight 12.0 kilos. Born 1928, 5 years old at death.
Liver and bread diet from weaning to maturity. Continuous anemia Dec., 1928,
to Dec., 1932.

Uneventful anemia history to *Aug. 5, 1930.* Vomiting. Weight 15.6 kilos.
Aug. 16, 1930. Urine: Albumin ++, granular casts, occasional red cell.
Intermittent periods of poor food consumption to Mar., 1931, otherwise history
uneventful.

TABLE 6

*Hemoglobin Production Shows Terminal Depression with Glomerulonephritis
and Uremia*

Dog 27-233.

Year	Pig liver	Beef muscle	Iron 21 mg.	Liver extract − iron	Liver extract + iron	Albumin in urine	Blood N.P.N.
	Net hemoglobin produced due to diet factors—*gm. per 2 wks.*						
							mg. per cent
1929			36	.			
1930	*68		31	46		++	
1931	103	33		44			
1932	77, 96			32	†75	+++	32
							200

Incomplete kidney feeding experiment 2 weeks before death shows zero hemo-
globin production.

* Reindeer liver contains 90 per cent as much potent material as pig liver (1).
† Death 3 months after completion of this experiment.

Mar. 16, 1931. Marked reaction to horse globin injection (2 gm.), severe
intoxication, recovery. Weight 15.3 kilos. *Mar. 24–26.* Occasional vomiting
unrelated to diet. *May–Dec.* Intermittent periods of decreased food consump-
tion.

Dec. 5. Marked diarrhea. Weight 14.8 kilos. *Dec. 5–11.* Left all food.
Sugar intravenously several doses daily. *Dec. 11.* Fibrin 620 mg. per cent.
Dec. 11–18. Several transfusions. Milk feeding by stomach tube. *Dec. 12.*
Gradually improving. Sugar intravenously, 2 to 3 doses daily, 12 days. Diar-
rheal stools contain blood clots. Vomiting. Transfusion. Weight loss 1.8
kilos 1 week. Temperature 38.3°. *Dec. 14.* Fibrin 475 mg. per cent. *Dec. 25.*
Recovery. Weight 11.8 kilos. Uneventful history to Mar. 14, 1932. *Mar.–
Aug., 1932.* Intermittent poor food consumption. Weight 15.5 kilos.

Aug. 22. Blood in feces. No diarrhea. Slight vomiting. *Aug. 23.* Urine: Albumin +++, no casts, no red cells. *Aug. 24.* Leucocytes 17,600. Stomach tube feeding. Sugar intravenously. *Aug. 31.* Leucocytes 11,600. Improving. Weight 14.1 kilos. *Sept. 24.* Diarrhea, blood in feces. *Sept. 30.* Hemoglobin value slight drop, 39 to 47 per cent. No blood removal for 6 weeks preceding. Weight 13.1 kilos. *Oct. 18.* Blood in feces. No parasitic ova. Left 90 per cent food. Urine: Albumin ++, granular casts, 10 to 20 red cells per field. Non-protein nitrogen 32 mg. per cent. *Oct. 18–Dec. 6.* History uneventful. Weight 12.5 kilos.

TABLE 7—*Summary*

Net Hemoglobin Production as Influenced by Nephritis

Test factor daily for 2 wks.	Control	Early nephritis	Late nephritis
Liver, 300 gm.			
Hemoglobin production, *gm.*	95	80	71
Per cent of normal	100	84 (14)	75 (9)
Liver extract without iron			
Hemoglobin production, *gm.*	50	47	36
Per cent of normal	100	94 (4)	72 (5)
Liver extract with iron			
Hemoglobin production, *gm.*	79	58	59
Per cent of normal	100	74 (4)	75 (7)
Kidney, 300 gm.			
Hemoglobin production, *gm.*	85	46	42
Per cent of normal	100	54 (2)	49 (8)
Iron, 40 mg.			
Hemoglobin production, *gm.*	55	53	41
Per cent of normal	100	96 (10)	75 (12)

Figures in parentheses indicate number of complete experiments.

Dec. 6. Kidney feeding experiment. *Dec. 15.* Dog sick. Food consumption 12 per cent. Vomiting. Non-protein nitrogen 200 mg. per cent. *Dec. 16.* Urine: Albumin ++, granular casts, numerous red cells. *Dec. 16, 1932.* Killed with ether. *Autopsy* done at once. Heart shows old endocardial scars in auricles and ventricles, the residue of vegetations. The mitral valve shows old healed vegetations—no recent vegetations anywhere. Lungs show patches of bronchopneumonia and edema in lower lobes. Kidneys show a few cortical scars. Other organs not related. Histological sections show subacute glomerulonephritis. The glomeruli show many adhesions to capsules. Mononuclears are numerous in stroma. Tubules are dilated and contain numerous casts.

Table 6 (dog 27-233) shows a continuous anemia history of 4 years. During this time the hemoglobin production on standard diets is within low normal limits. Renal injury was first noted in 1930. An intestinal infection was contracted in December, 1931, and caused severe clinical intoxication with bloody diarrhea. A second episode of diarrhea and intoxication was noted in August, 1932. The etiology of these infections was not determined but it was probably due to one of the dysentery group of bacteria. No determinations of hemoglobin production were made during these periods of dysentery and convalescence. 10 days before death a kidney feeding experiment was begun. For a week the food consumption was satisfactory and the hemoglobin production zero but it is not safe to say that the nephritis alone was responsible for this as autopsy showed a terminal bronchopneumonia which may well have been a factor.

Summary Table 7 needs little comment.

In addition to the five dogs tabulated above, determinations from five other dogs with fatal nephritis are included. These ten dogs with glomerulonephritis were observed in a group of 92 standard anemic dogs coming to autopsy in the last 10 years. The division into early and late nephritis was arbitrary and usually each period represented one-half of the life story after renal injury was established. In the early nephritis group we see that the averages of hemoglobin production run close to the control hemoglobin output for normal dogs—about 85 per cent of normal if we exclude the figures on kidney feeding because of too few experiments. The late nephritis averages are a little lower (70 per cent of normal) but this drop is not conspicuous nor uniform.

SUMMARY

Spontaneous glomerulonephritis develops not infrequently (11 per cent incidence) in the anemia colony. The course of the nephritis is insidious and usually extends over several years but ends in uremia, often with terminal bronchopneumonia.

Hemoglobin production in these standard anemic dogs is well established as related to various standard food factors. These tests are summarized in the tables above to show the changes that appear year by year in the life of each dog.

Nephritis causes little or no change in hemoglobin production in anemic dogs in the early stages of the disease. In the late stages of nephritis there may be no change or moderate changes in hemoglobin production in these anemic dogs. The average is 70 per cent of normal hemoglobin production in advanced nephritis.

It seems unlikely that this degree of impairment of hemoglobin production in nephritis would result in *spontaneous anemia* in the dog.

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THE ANTIBODY RESPONSE TO SWINE INFLUENZA*

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It is well known that antibodies capable of neutralizing swine influenza virus are present in the sera of swine recovered from swine influenza (1–4). However, neither the exact time following infection that these antibodies first appear nor the time at which they reach their highest titer has been determined. The present experiments were conducted in order to obtain this information.

Methods

Five swine were inoculated intranasally with a mixture of strain 15 swine influenza virus (1) and *Hemophilus influenzae suis* (5). Four of these animals developed typical swine influenza, while the 5th had an extremely mild illness like the "filtrate disease" seen in swine infected with virus alone (1). A 6th pig was inoculated intranasally with swine influenza virus alone and developed filtrate disease. These 6 swine were bled just prior to infection and then repeatedly during illness and after recovery, and the sera thus obtained were titrated for antibodies which neutralize swine influenza virus.

Titration of Antibodies for Swine Influenza Virus.—The neutralization tests were conducted in the usual way in white mice (3), employing the supernatant of a 2 per cent suspension of glycerinated infected mouse lung as virus and mixing this in equal parts with the serum dilution to be tested. Strain 15 swine influenza virus was used in all tests. The serum dilutions were prepared in physiologic salt solution, using 0.2 cc. of serum in varying amounts of the diluent. A further twofold dilution occurred, when the serum was added to the virus suspension. Three etherized mice were inoculated, in testing each serum dilution, by dipping their noses in the virus-serum mixture contained in a slightly tilted small Petri dish. Each neutralization experiment was allowed to run for 10 days. Mice which succumbed during the 10 day observation period and showed typical pul-

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monary pathology of influenza at autopsy were considered to have received a non-neutralizing dilution of serum. Those which survived the 10 day period were considered to have received a neutralizing dilution of serum. The final titer of a given serum was taken as the highest dilution which protected all or the majority of the mice against death.

RESULTS

Swine 1993 (Fig. 1) developed typical swine influenza of 7 days' duration, and neutralizing antibodies were first detectible in its serum on the 6th day after infection. They rose to a titer of 1:20 on the 7th day and remained at this level until the animal was killed on the 11th day.

Swine 1974 (Fig. 2) was ill of swine influenza for 7 days following inoculation, and neutralizing antibodies first appeared in the serum on the last day of illness. By the 10th day the antibody titer had reached 1:20, and a titer of 1:60 was attained on the 14th day. Two days later it had decreased to 1:40, and this level was maintained until the termination of the experiment on the 84th day.

Swine 1975 (Fig. 3), inoculated in the same manner with a mixture of swine influenza virus and *H. influenzae suis*, did not show the usual clinical manifestations of swine influenza. Instead it underwent only a brief mild illness characterized clinically by malaise and inappetence of 2 days' duration. The clinical picture was indistinguishable from filtrate disease seen in swine infected with swine influenza virus alone. Infections of this type are of extremely rare occurrence in fully susceptible swine. The exact time at which neutralizing antibodies first appeared in the serum of this pig is unknown, because bleedings were unfortunately omitted on the 8th and 9th days after inoculation. No antibody was detectible on the 7th day, while by the 10th day the titer had reached 1:40. By the 14th day the titer had risen to 1:60, and this level was maintained through the 21st day. On the 27th day the antibody titer was found to have risen to 1:120, and this level was maintained through the 46th day. It had decreased to 1:80 on the 62nd day and was still 1:80 on the 84th day when the experiment was terminated.

Swine 1984 (Fig. 4) was ill of swine influenza for 6 days, and neutralizing antibody first became detectible in its serum on the 7th day. By the 10th day the titer had risen to 1:20 and on the 14th day it reached 1:40. By the 20th day the antibody titer was 1:120. At this time the animal was tested for active immunity to swine influenza by intranasal inoculation with a mixture of swine influenza virus and *H. influenzae suis*. It proved solidly immune. Serum was obtained 6, 12, 24, 50, and 72 hours following the immunity test and titrated for neutralizing antibody, but no significant change in the antibody titer was observed. The titer was still 1:120 when the experiment was terminated on the 31st day.

Swine 1985 (Fig. 5) had a characteristic swine influenza of 7 days' duration, and neutralizing antibody first appeared in the serum on the last day of illness. The titer reached 1:20 on the following day and by the 10th day had risen to 1:80.
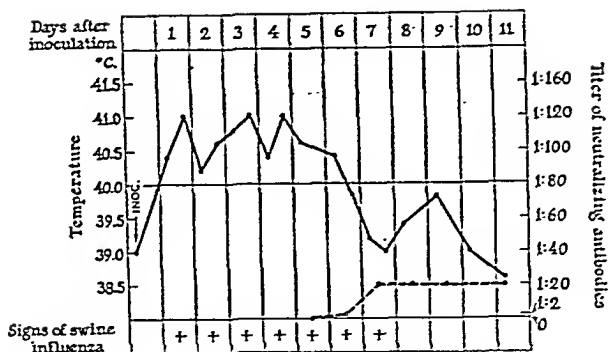
FIG. 1. Swine 1993. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*

Figs. 1 to 6. The full line represents temperature; the broken line, neutralizing antibody titer.
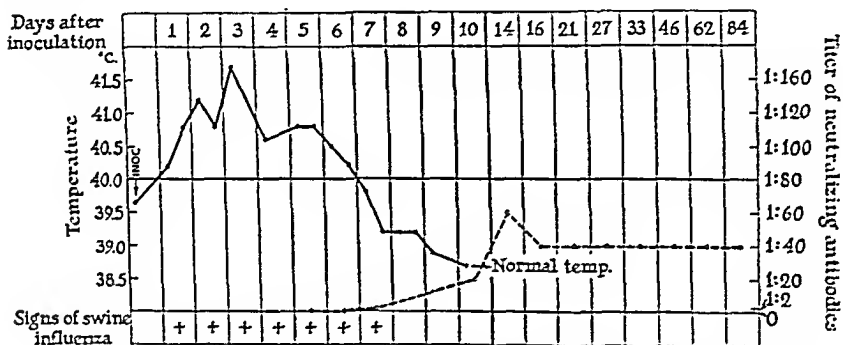
FIG. 2. Swine 1974. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*
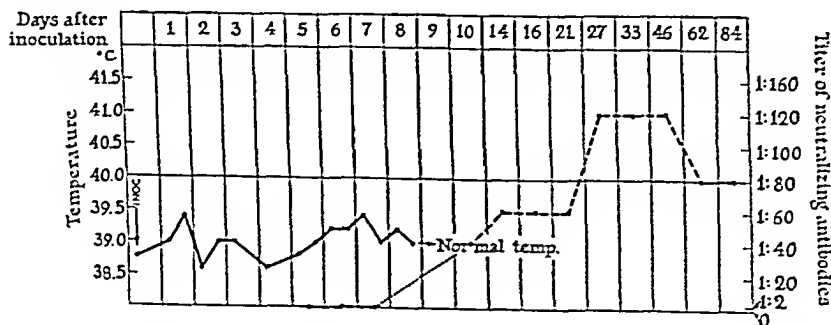
FIG. 3. Swine 1975. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*
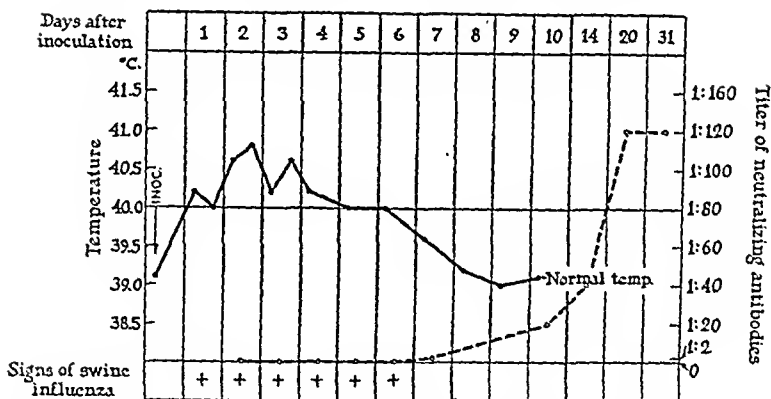
FIG. 4. Swine 1984. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*
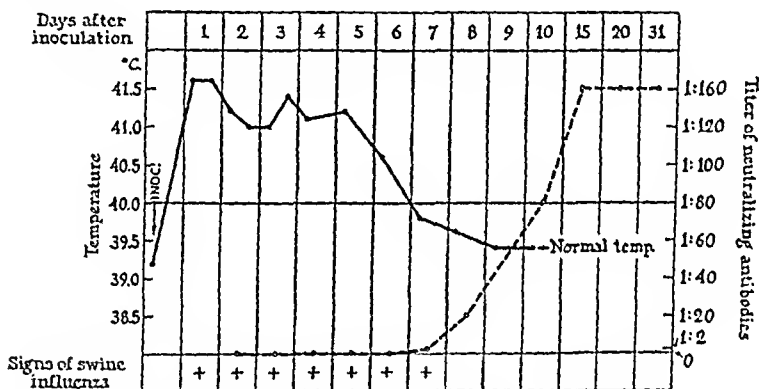
FIG. 5. Swine 1985. Inoculated intranasally with swine influenza virus + *H. influenzae suis.*
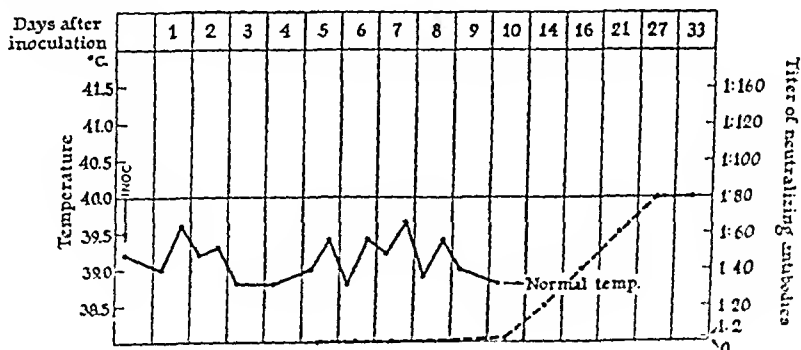
FIG. 6. Swine 2002. Inoculated intranasally with swine influenza virus alone.

On the 15th and 20th days the antibody titer was 1:160.   The animal was tested for active immunity to swine influenza on the 20th day and found to be solidly immune.   Serum drawn 6, 12, 24, 50, and 72 hours after the immunity test was titrated for antibody, but no significant change was found.   The titer remained at 1:160 when the experiment was concluded on the 31st day.

Swine 2002 (Fig. 6) was infected with swine influenza virus alone and underwent an attack of the mild filtrate disease.   There was no significant temperature elevation, and clinically the illness was characterized by malaise and inappetence of 2 days' duration.   Neutralizing antibody first became detectible in the serum of this animal on the 10th day.   The antibody titer rose gradually to 1:80 on the 27th day and persisted at this level on the 33rd day when the experiment was terminated.
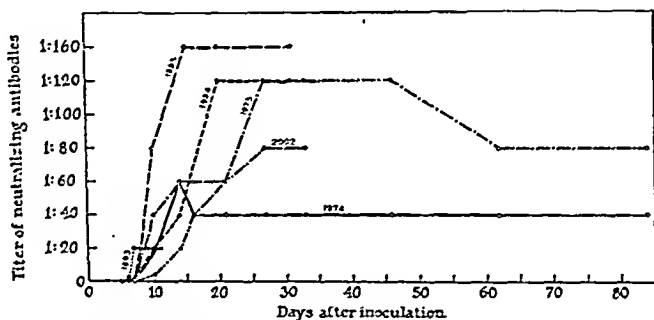
FIG. 7. Comparative antibody response of the 6 swine to swine influenza.

DISCUSSION

The results summarized in Fig. 7 illustrate the variable extent of the antibody response of individual swine to swine influenza.   In the 4 animals that underwent typical attacks of the disease, antibodies were present by the 7th day after infection, while in the 2 that suffered only the mild filtrate disease, antibodies did not appear until sometime after the 7th day.   Similarly the time required to reach the highest antibody titer appeared to be influenced by the clinical severity of the disease.   Excluding swine 1993, observed for only 11 days, the animals with typical influenza reached their maximum titers on the 14th, 15th, and 20th days after infection.   The 2 swine that underwent attacks of the mild filtrate disease, on the other hand, did not reach their maximum titers until the 27th day after infection.   There was no apparent relationship between clinical severity of disease and the

maximum antibody titers eventually reached. These ranged from 1:60 to 1:160. In 2 animals kept under observation for 84 days there was some decrease in titer from the highest level attained.

The present findings concerning the antibody response in swine influenza are similar to those noted by investigators of human influenza. Smith and Stuart-Harris (6) observed that the antibody titer of a human case had risen considerably by the 8th day after onset of illness, reached a peak between the 16th and 31st days, and had declined slightly by the 44th day. Francis and his coworkers (7) noted, in another human case, that the antibody titer rose abruptly on the 7th day, reached a peak on the 14th day, and then gradually declined. Smorodintseff and his coworkers (8) reported 25 to 100-fold increases in the neutralizing antibody titers of the sera of their volunteers 10 to 15 days after experimental infection with human influenza virus.

In the 4 swine that underwent typical attacks of swine influenza, the appearance of neutralizing antibodies coincided rather closely with defervescence and clinical recovery, suggesting that the antibodies may have contributed materially to the cessation of signs of illness. It is known, furthermore, that swine influenza virus is, as a rule, no longer demonstrable in the swine respiratory tract 7 or more days after infection. The anatomical changes produced in the lung by the infection, however, persist for a variable period of time after recovery is clinically apparently complete. The possibility that the appearance of circulating virus-neutralizing antibody is the sole cause of clinical recovery is rendered unlikely by the findings in the cases of the 2 mildly ill animals, in which, though signs of clinical infection persisted for only 2 days, neutralizing antibody did not become detectible until after 7 days.

SUMMARY

Antibodies that neutralize swine influenza virus became detectible in the serum of swine on the 6th or 7th day after infection with swine influenza. Their appearance corresponded rather closely with clinical recovery. In swine with the milder filtrate disease, neutralizing antibodies did not appear until sometime between the 7th and 10th days. The maximum antibody titers ranged from 1:60 to 1:160 and were attained on from the 14th to the 27th days after infection.

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VITAMIN C IN RELATION TO EXPERIMENTAL POLIOMYELITIS

WITH INCIDENTAL OBSERVATIONS ON CERTAIN MANIFESTATIONS IN MACACUS RHESUS MONKEYS ON A SCORBUTIC DIET

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PLATES 30 AND 31

Data have recently been presented to show that the administration of natural vitamin C during the incubation period of experimental poliomyelitis is capable of modifying the course of the disease and of preventing paralysis in a considerable number of *Macacus rhesus* monkeys. ·

With the Aycock strain of the virus and the intracerebral route of inoculation, Jungeblut (1, 2) found that 77 of 243 (31.3 per cent) vitamin C-treated monkeys escaped paralysis, as compared with only seven of 136 (5.2 per cent) untreated animals. When synthetic vitamin C was used, no significant effect was obtained since only eleven of 101, or 10.2 per cent, failed to develop paralysis. With the M.V. virus (special strain used exclusively for nasal instillation for several years and obtained from the writer) instilled intranasally Jungeblut (2) reported that only one of twenty monkeys treated with 5 to 25 mg. of natural vitamin C daily (optimum dose in intracerebral experiments) showed no paralysis, while among ten animals treated with 50 to 100 mg. daily nine escaped paralysis; fifteen untreated monkeys all became paralyzed.

Since any substance exerting such an effect on experimental poliomyelitis might be a most useful therapeutic agent, it was deemed desirable to determine whether or not these results could be reproduced.

Methods

The strain of M.V. virus, the technique of nasal instillation, and other details which contribute to the constancy of infection (nearly 100 per cent in over 100 monkeys) by the nasal route were described in a recent communication (3).

The infecting dose was 1 cc. of a 5 per cent suspension of virus for each nostril on two occasions at an interval of 4 to 6 hours. When the second dose is omitted the number of monkeys which succumb may be reduced by half, indicating that the dosage employed is just within the range of that required to infect successfully all monkeys (provided the virus is fresh or has not been in glycerol much longer than a month). Two preparations of vitamin C, natural and synthetic,[1] were used. The vitamin was dissolved in distilled water just before using and the injections were given subcutaneously. When 400 mg. of the synthetic crystalline preparation was administered in a single daily dose, it was neutralized with NaOH just before using. The vitamin was administered immediately after nasal instillation of the virus and once daily thereafter until the outcome was clearly apparent.

Effect of Vitamin C on Disease Induced by Nasal Instillation of Virus

Thirty-six monkeys were used in the first test. They were on a vitamin C-adequate diet (oranges, bananas, bread, and milk) for at least 9 days before virus instillation, during which time their rectal temperatures were recorded daily as an aid in the interpretation of any subsequent febrile reaction. All monkeys were instilled at the same time with a pool of poliomyelitic cords which had been kept in 50 per cent glycerol for 3 months. Virus of this age was used with the hope that less than 100 per cent of the control animals would succumb. Ten monkeys were untreated; six received at the time virus was first instilled and daily thereafter 5 mg. of natural vitamin C; ten, 100 mg. of natural C; and ten, 100 mg. of synthetic, crystalline cevitamic acid.

The results recorded both in Chart 1 and Table I show that vitamin C neither modified the course of the disease nor prevented paralysis: 80 per cent of the untreated monkeys and 90 per cent of the treated ones developed paralysis. With these data at hand, Dr. Jungeblut's advice was sought and a similar experiment was carried out jointly in his laboratory. In a group of forty monkeys, among which ten were controls and thirty were treated with varying amounts of vitamin C, only one monkey, a treated one, escaped paralysis.

Influence of Preliminary Diet

In an attempt to find an explanation for the discrepancy between the present and the reported (1, 2) results, it appeared that vitamin C may perhaps be effective only at the time it is being taken up by depleted tissues. If that were the case, one might expect no effect in monkeys which had been in the laboratory on an adequate vitamin

[1] Kindly supplied by Merck and Company.

−10 No signs of poliomyelitis
Received virus again 7 weeks after first instillation and developed typical poliomyelitis
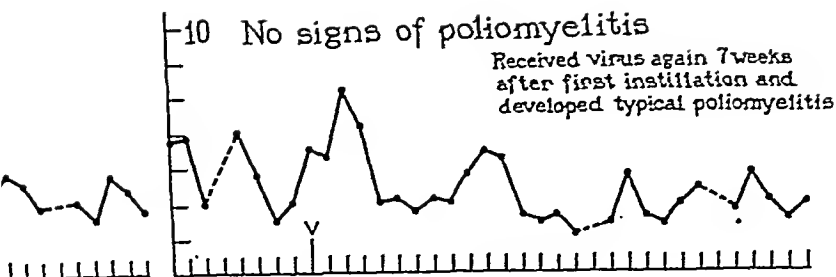
. − Paralysis of both legs
+L.−   "   " arms and legs
: −   "   " facial muscles
− Partial paralysis
? − Complete   "
− Weakness
− Ptosis of eye-lids

−26 No signs of poliomyelitis
Received virus again 5 weeks after first instillation and developed typical poliomyelitis

−36 No signs of poliomyelitis
Received virus again 7 weeks after first instillation and developed typical poliomyelitis
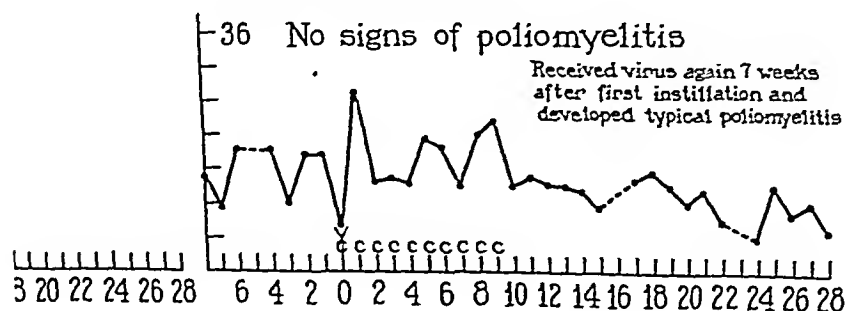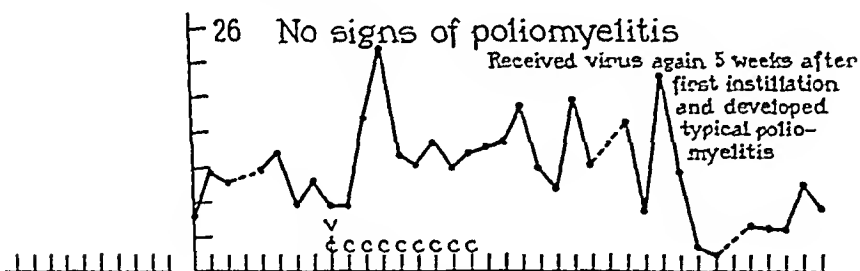
C diet for some time prior to the experiment (as was the case in the present tests), while in monkeys whose store of vitamin C was depleted a beneficial result may perhaps be obtained. This possibility was submitted to experiment.

Forty-six monkeys, selected for their excellent physical condition and nutrition, were obtained in a single lot on Jan. 4, 1938, from one dealer who stated that

TABLE I

Effect of Vitamin C on Experimental Poliomyelitis

Series	Preliminary diet	Vitamin C given daily following nasal instillation of virus	Number of monkeys	Number developed paralysis
I	Vitamin C *adequate* for at least 9 days before test	None	10	8
		5 mg. natural	6 (1 died without evidence of poliomyelitis)	5
		100 " "	10	9
		100 " synthetic	10	9
II	Vitamin C *adequate* for at least 21 days before test	None	6	6
		100 mg. natural	7	7
		400 " synthetic	7	7
	Vitamin C *deficient* for 21 days before test	None	5	4
		100 mg. natural	7	7
Total untreated.............................			21	18 (86 per cent)
Total treated...............................			47	44 (94 per cent)

they had arrived in a single shipment on Dec. 28, 1937, and that during the voyage, as well as in his own establishment, their diet included oranges and other fresh fruits. Twenty-one of these monkeys were placed on the regular laboratory diet (bread, pasteurized milk, oranges, and bananas) and at the end of 3 weeks only one died, the remaining twenty being healthy and well nourished. The other twenty-five monkeys were put on the following vitamin C-inadequate diet:

Milk (boiled with a constant stream of compressed air for 1 hr.)   250 cc.

Granulated sugar   15 gm.
Cod liver oil   15 cc.
Whole wheat bread   80 gm.
Peanuts   *ad lib.*

The indicated amounts were those allowed daily for each monkey. The diet was only partly consumed due to the monkeys' dislike for cod liver oil and when it was found that they were not thriving, it was changed on the 12th day to pasteurized whole milk with added cane sugar and 2 drops of drisdol (crystalline vitamin D in propylene glycol) per monkey, white bread, and peanuts; the new diet was completely consumed. At the end of 3 weeks thirteen of the twenty-five monkeys had died in a manner to be described in a subsequent section.

On Jan. 25, 1938, *i.e.*, at the end of 3 weeks on their respective diets, all the monkeys were given virus intranasally. Of the twenty which had been on the vitamin C-adequate diet, six served as controls, seven received 100 mg. of natural vitamin C daily, and seven others were given 400 mg. crystalline synthetic vitamin C daily at Dr. Jungeblut's suggestion, who believed from his unreported experiments that the synthetic vitamin might be effective when administered in larger amounts. Of the twelve remaining monkeys which were on the vitamin C-deficient diet, five served as controls and seven were given 100 mg. of natural vitamin C daily.

The results recorded in Table I and Chart 2 show no effect of vitamin C in either group. Of the thirty-two monkeys all but one developed paralysis and that one was in the untreated control group which had been fed with the vitamin C-deficient diet.

Some Manifestations in Monkeys on a Scorbutic Diet

Since hitherto few monkeys have been used in vitamin C studies, it may be of interest to record some of the more striking manifestations among the *rhesus* monkeys which were on a scorbutic diet in the present investigation. That which attracted most attention was the death of thirteen of twenty-five monkeys between the 9th and 18th days after they had been put on the scorbutic diet, while among the twenty-one monkeys on the regular diet all remained well during the same period.

Postmortem examination revealed evidence of acute infections in most of the fatal instances—five had non-tuberculous, lobar pneumonia with complete consolidation of one or more lobes; three, hemorrhagic enterocolitis; one, an erysipelas-like infection of the face; and in four there were no gross pathological changes other than emaciation; the monkeys that died with pneumonia were all well nourished (see Chart 3). While none of the monkeys showed any definite clinical or gross pathological evidence of scurvy, there was, nevertheless, microscopic evidence of early scurvy in the ribs and lower ends of the femurs of some of them.
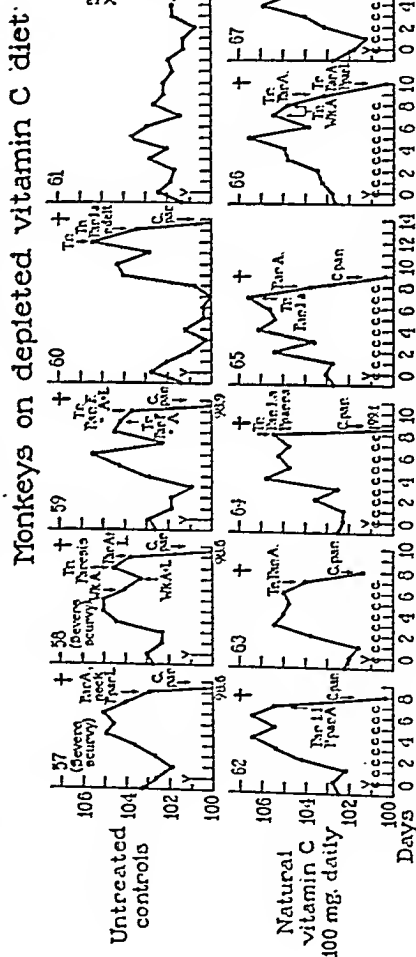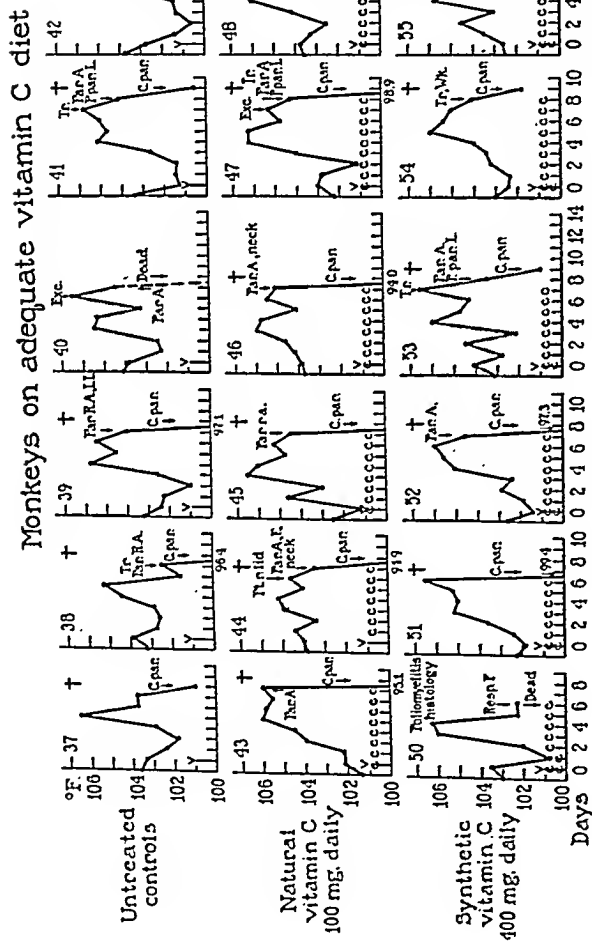
CHART 2. Influence of preliminary diet on subsequent effect of subcutaneous injections of vitamin C on the course of experimental poliomyelitis.

The increased susceptibility to spontaneous infection among guinea pigs on scorbutic diets and among human beings with scurvy has been noted by several investigators. It is also interesting that the infections observed by Höjer (4) and others in guinea pigs on vitamin C-deficient diets were chiefly of the upper respiratory tract and pneumonia, septicemia, and enterocolitis. The present observations on monkeys, which correspond so closely to those on guinea pigs, derive special significance from the practically total absence of acute infec-

21 monkeys on vitamin C-adequate diet

25 monkeys on scorbutic diet

Days on diet

☒ –Lobar pneumonia, non-tuberculous
– Enterocolitis
– No gross pathological change other than emaciation
⫼ –Erysipelas-like infection of face
☒ –Pulmonary tuberculosis

Each square represents one dead monkey
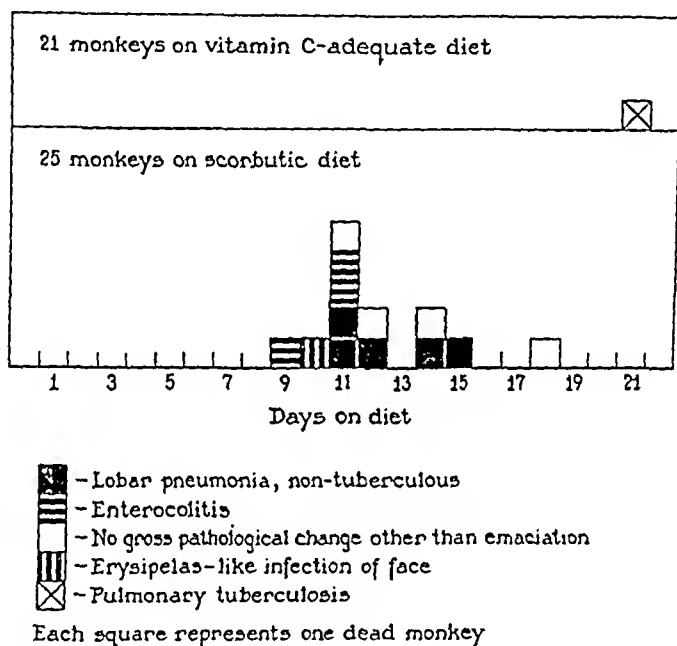
CHART 3. Mortality in relation to diet in a single colony of *rhesus* monkeys.

tion among the other twenty-one monkeys of the same group which were receiving the regular diet.

The twelve remaining monkeys all received poliomyelitis virus 3 weeks after the beginning of the vitamin C-deficient diet. Clinical, x-ray, or postmortem evidence of scurvy was found among three of the five monkeys whose diet was not supplemented by vitamin C, but not among the seven which received 100 mg. of natural vitamin C daily.

Two of these three showed their first signs of scurvy at the end of the 4th week after the beginning of the diet and during the course of poliomyelitic infection.

Monkey 58 first exhibited edema of the eyelids which became discolored the following day from spontaneous hemorrhages; there were also some sponginess and subgingival hemorrhage about three teeth. While there was no other external hemorrhage, the tourniquet test applied to the upper extremity yielded a large crop of petechiae on the arm and forearm distal to the tourniquet. There was a peculiar clumsiness of the upper and lower extremities (recorded as weakness) for at least 2 days before the onset of the characteristic nervous signs of poliomyelitis, apparently as a result of subepiphyseal fractures which were found at autopsy. The lower ends of both femurs and the upper ends of both tibiae and humeri showed varying degrees of fracture, periosteal separation, and subperiosteal hemorrhage (Figs. 1 and 2). "Mushrooming" of some of the ribs at the costochondral junctions could be discerned by palpation, as well as by x-ray, which also revealed "cupping," loss of calcium, and focal trabecular disintegration (Figs. 4 and 5). Outside the skeleton there was a striking reaction in the abdomen where there was considerable hemorrhage in the wall beneath the parietal peritoneum as well as large, subcapsular blood clots around both kidneys (Fig. 3). The writer has not found reference to hemorrhages in these sites and it is quite probable that they were the result of the method used in catching the monkeys, which involves grasping the animal by the small of the back. Monkey 57 showed gross changes only in the lower ends of both femurs (epiphyseal infractions) and by x-ray in some of the ribs. Monkeys 59 and 60 showed no gross scorbutic changes. Monkey 61 exhibited no evidence of poliomyelitic infection. On the 42nd day after the beginning of the diet, it had questionable gingival changes and also some difficulty in climbing; on the 43rd day both the upper and lower eyelids were edematous, and the gums were red and spongy about the incisor teeth. 2 days later the eyelids were hemorrhagic; it had lost the use of its right arm and could climb only with the greatest difficulty. The tourniquet test was positive on two occasions; it was negative in six normal monkeys. The monkey appeared ill, was disinclined to move, but it was quite evident that the loss of function in the extremities was due to spontaneous fractures—bony crepitus was clearly elicited—rather than to nervous involvement. X-ray revealed fractures with displacement at the lower ends of both femurs (Figs. 8 and 16); scorbutic changes were also apparent in the upper ends of the humeri and in the ulna at the wrists (Figs. 12 and 14). On completion of the 4 weeks of observation following the instillation of virus (i.e., 49 days of deficient diet), 1 gm. of the crystalline, synthetic cevitamic acid used for treatment of the other monkeys was given subcutaneously to monkey 61. The following day the hemorrhage and edema of the eyelids had disappeared, the tourniquet test was negative, and the monkey appeared much improved. Smaller doses of vitamin C continued to be given daily and within 4 to 5 days it regained the use of its extremities and x-rays taken 10 days later showed repair of the fractures with calcification of what must have been extensive subperiosteal hemorrhages about the femurs, humeri, one ulna, and costochondral junctions (Figs. 7, 9, 11, 13, 15, and 17). These x-ray findings were confirmed at autopsy.

In correlating the manifestations in monkeys with the changes seen in human scurvy, it should be noted that the age of the monkeys used in these studies is equivalent to that of 10 to 13 years in human beings calculated on the basis of the development of ossification centers and the open epiphyses. The bony changes observed here correspond closely to those seen in scorbutic children. In so far as the high incidence of acute infections can be attributed to the inadequate diet, it is noteworthy that the increased susceptibility to spontaneous infection developed before any gross signs of scurvy had appeared. The impression was gained that in the investigation of certain problems relative to vitamin C deficiency *rhesus* monkeys might be used more advantageously than guinea pigs.

DISCUSSION AND SUMMARY

In the experiments reported in the present communication it was found that vitamin C, both natural and synthetic preparations, had no effect on the course of experimental poliomyelitis induced by nasal instillation of the virus. The objection cannot be raised that too large an amount of virus was used, since recent studies (3) on the fate of the nasally instilled virus indicated that all but an undetectable amount of it is swallowed and disappears from the nasal mucosa within 3 hours or less, and that none is demonstrable in the central nervous system before the 3rd day. Vitamin C administration was begun immediately after the instillation of virus and if it were capable of exerting any effect on the virus or the tissues it could have done so even before multiplication of virus had begun. Monkeys whose store of vitamin C was depleted reacted in the same way as those receiving an adequate diet. There is no apparent explanation for the difference between these results and those reported earlier by Jungeblut (1, 2).

During the present investigation it was found that monkeys on a scorbutic diet died of spontaneous acute infections, chiefly pneumonia and enterocolitis, while their mates receiving an adequate diet remained well. The surviving monkeys on the scorbutic diet developed the osseous and other changes of human scurvy, and the vitamin C used in this study was shown to produce healing and calcification in the bones as well as to check the edema and hemorrhagic diathesis.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Lower end of femur of scorbutic monkey (M 58). Arrows point to subepiphyseal fracture and subperiosteal hemorrhage. × 2.

FIG. 2. Upper end of tibia of M 58. Note separation of periosteum from shaft without hemorrhage, in addition to subepiphyseal fracture. × 2.

FIG. 3. Subcapsular perirenal hemorrhage in M 58. × 2.

(Sabin: Vitamin C in relation to poliomyelitis)

PLATE 31

Fig. 4. Ribs of normal monkey. Note the almost straight line between the bone and cartilage. × 2.

Figs. 5 and 6. Ribs of two scorbutic monkeys. Arrows point to "mushrooming" and "cupping" of costochondral junction and to foci of trabecular disintegration. × 2.

Fig. 7. Ribs of scorbutic monkey (M 61) 10 days after vitamin C. Note rosary formed by calcification of subperiosteal hemorrhage in all the fixed ribs with the exception of the upper three, suggesting the rôle of the respiratory movements in the causation of these changes. Actual size.

Fig. 8. Right knee of M 61 just before vitamin C therapy. Arrow points to fracture and displacement at lower end of femur; note also the light zone of diminished calcification.

Fig. 9. Same knee 10 days after beginning of vitamin C therapy. Arrow points to calcified subperiosteal hemorrhage.

Figs. 10 and 11. Right humerus (M 61) before and 10 days after vitamin C. Arrows in Fig. 11 point to calcified subperiosteal hemorrhage.

Figs. 12 and 13. Right wrist (M 61) before and 10 days after vitamin C. Arrow points to the more marked zone of decalcification in the ulna prior to vitamin C.

Figs. 14 and 15. Left wrist (M 61) before and 10 days after vitamin C. Arrow in Fig. 15 points to calcified subperiosteal hemorrhage.

Figs. 16 and 17. Left knee (M 61) before and 10 days after vitamin C. Arrow in Fig. 16 points to fracture with displacement in femur and that in Fig. 17 to calcified subperiosteal hemorrhage.

THE SOLUBLE MALARIAL ANTIGEN IN THE SERUM OF MONKEYS INFECTED WITH PLASMODIUM KNOWLESI

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When *rhesus* monkeys with acute *Plasmodium knowlesi* infection recover after administration of quinine, complement-fixing antibodies are present at maximum titer in the serum soon after the first acute infection has subsided (1), and protective (2) and agglutinating (3) antibodies are demonstrable somewhat later in the course of the chronic disease. Relapses cause an increase in the titer of complement-fixing antibodies. From previous studies it has been impossible to learn whether the antigens and antibodies concerned in the complement fixation reaction are identical with or different from the antigens and antibodies concerned in active immunity and passive protection.

The results of the present work show that a soluble malarial antigen occurs in the serum of monkeys with acute *Plasmodium knowlesi* infection. This antigen, upon injection into normal monkeys, gives rise to complement-fixing antibodies which are similar to those produced by malarial infection, but no protective or agglutinating antibodies are formed. This paper will describe serological and chemical investigations of the soluble malarial antigen.

Materials and Methods

The sera to be tested for soluble malarial antigen were obtained by allowing the parasitized blood to clot, or by collecting the blood in an equal volume of 2 per cent sodium citrate solution. In either case the serum was separated from the red cells as soon as possible after drawing the blood in order to minimize the possibility of extraction of antigen from the parasites *in vitro*. Chloroform was added as a preservative. The preparations containing sodium citrate were anticomplementary, but these could be made suitable for use in the complement fixation test by dialyzing them for 24 hours and then centrifuging to remove

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the precipitated fibrin and globulins. Removal of the fibrin was also accomplished by adding calcium chloride solution in order to bring about clotting of the citrated plasma, shaking with glass beads, and centrifuging. A concentration of about 0.5 per cent calcium chloride was required.

Immune sera were obtained from monkeys with chronic *P. knowlesi* infection. Some of these animals had been repeatedly superinfected with large doses of living parasites. The same sera had been tested by complement fixation with antigens from parasitized red cells or spleens (1) and by agglutination (3).

The method of performing the complement fixation test was identical with the procedure described previously (1), except that serum from monkeys with acute *P. knowlesi* infection was used in place of malarial antigens extracted by saline from parasitized blood cells or spleens. To distinguish this from blood antigen and spleen antigen the antigen-containing serum will be referred to in this paper as serum antigen.

For the immunization of monkeys the citrated plasma from heavily parasitized blood was injected intravenously. In order to exclude the possibility of injecting living parasites, the serum antigen was treated with chloroform, allowed to stand in the ice box for at least 3 days, and centrifuged at 3000 R.P.M. for half an hour before it was injected into the monkeys. After a longer time in the ice box these preparations formed a fibrin precipitate, and it was found necessary to centrifuge them again at 3000 R.P.M.

Detection of the Soluble Malarial Antigen

Strong complement fixation reactions were regularly obtained when the sera of monkeys with severe, acute *P. knowlesi* infection (as antigens) were tested with antiserum from a hyperimmunized monkey. Sera from fourteen monkeys with parasite counts above 500 per 10,000 red blood cells all gave complement fixation up to dilutions of 1:32 to 1:128 with antiserum diluted 1:10. Sera from eight monkeys with chronic malaria and parasite counts below 10 per 10,000 red blood cells gave no complement fixation with the same antiserum. No reaction was obtained when sera from normal monkeys were tested with the immune serum.

The soluble malarial antigen may fail to give a definite fixation of complement with certain immune sera from monkeys with chronic *P. knowlesi* infection. Of fourteen immune sera tested against a single preparation of serum antigen, nine gave strong fixation of complement, two gave weak reactions, and three gave negative reactions. The sera which gave weak or negative reactions with the serum antigen also showed low titers of complement-fixing antibodies when tested

with antigen prepared from parasitized red cells. Sera from ten normal monkeys gave no fixation of complement with the serum antigen.

Relation of the Soluble Malarial Antigen in the Serum to the Parasite Count

The sera of two monkeys were collected every 24 to 48 hours during acute infection with *P. knowlesi*. The titer of soluble antigen in these sera was determined by complement fixation with an immune serum (diluted 1:10) from a monkey with chronic malaria. The results are
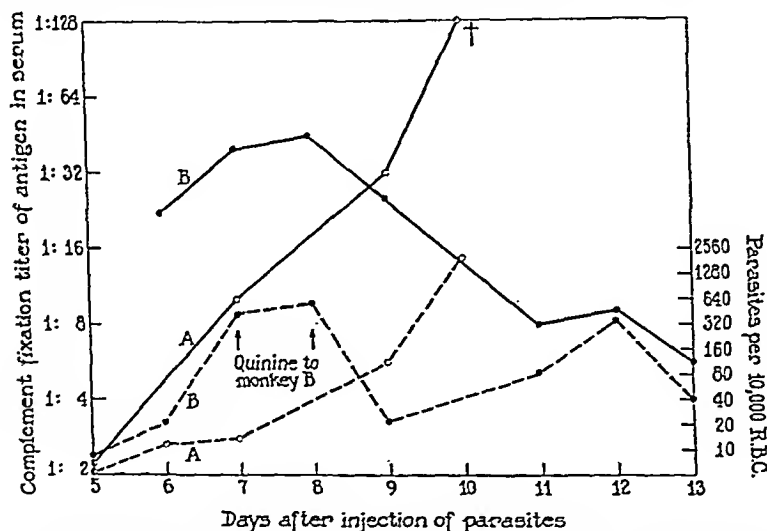
FIG. 1. Relation of soluble malarial antigen to parasite count in acute infection with *P. knowlesi*. Solid lines represent titer of antigen, broken lines parasite count.

presented graphically in Fig. 1. Monkey A received no treatment and died on the 10th day. The rapid rise in the parasite count of this animal was accompanied by a corresponding rise in the titer of soluble antigen between the 5th and 10th days after inoculation. Monkey B was treated by intramuscular injection of 3 cc. of a 3 per cent solution of quinine on the 7th and 8th days and subsequently recovered. The initial rise of the parasite count was accompanied by a rise in the titer of antigen in the serum, and after the injection of quinine the

parasite count and titer of soluble antigen both fell together. However, the secondary rise in the parasite count between the 9th and 12th days was not accompanied by an increase in the amount of soluble antigen in the serum. At this time the monkey was beginning to develop active immunity to the infection.

The disappearance of the soluble malarial antigen from the serum, after the acute infection subsides, is indicated by the results presented in Table I. For example, blood B-1, taken at 6 days, showed a para-

TABLE I

Relation of Titer of Serum Antigen to Parasite Count at Various Times during Plasmodium knowlesi Infection

Blood No.	Time after inoculation	Parasites per 10,000 red cells	Titer of antigen in serum
	days		
A-1	7	16	1:8
A-2	9	121	1:32
B-1	6	21	1:16
3	6	198	1:32
4	14	740	1:64
B-2	13	40	1:4
5	14	510	1:64
F-1	21	664	1:32
6	27	6	Trace
G-1	32	6	0
F-2	33	6	0
7	32	35	Trace
8	62	50	0
G-2	120	335	1:8
G-3	123	150	0

site count of 21 and a titer of antigen of 1:16, while blood B-2, taken from the same monkey at 13 days, had a parasite count of 40 and an antigen titer of only 1:4. When the infection became chronic, no soluble antigen was demonstrable in the serum, although the parasite count was as high as it was at the beginning of the acute infection. This may be seen from a comparison of A-1, A-2, B-1, and 3 with 7, 8, and G-3 in Table I. During a relapse when the parasite count was very high, soluble antigen reappeared in the serum (blood G-1, G-2, G-3), but milder relapses were not accompanied by the appearance of detectable amounts of antigen.

Reaction of Serum Antigen with Malarial Sera from Human Beings

Complement fixation reactions of human malarial sera with an antigen prepared from the parasitized red cells of monkeys dying of acute *P. knowlesi* infection have been described previously (5). The results of performing similar tests with serum antigen in place of antigen from parasitized blood are presented in Table II. The results indicate that the reactions obtained with human sera and serum antigen from monkeys were generally weak or doubtful, although the

Complement Fixation with Human Sera and Antigens from Serum and Parasitized Cells of Monkeys Dying of Plasmodium knowlesi Infection

Serum	Dilution	Serum antigen 1:16	Normal monkey serum 1:16	Antigen from parasitized blood 1:16	Antigen from normal blood 1:4
Malarial human					
Lc	1:4	++	0	++++	+
Rl	1:4	±	0	++++	0
Pn	1:4	0	0	++++	0
Jn	1:4	±	0	++++	0
Rd	1:4	+	0	++++	0
Ir	1:4	+	0	++++	0
Normal human					
Rn	1:4	0	0	0	0
Hn	1:4	0	0	0	0
Er	1:4	0	0	0	0
Dn	1:4	0	0	0	0
Immune monkey	1:4	++++	x	++++	0
Immune monkey	1:4	++++	x	++++	0
Normal monkey	1:4	0	x	0	0

x = not done.

same human sera gave strong reactions with antigen from parasitized blood, and immune monkey sera gave equally strong reactions with both serum antigen and parasite antigen.

Tests for Cross Reactions with Soluble Antigens from Filterable Viruses

Soluble antigens have been described for the viruses of yellow fever (6), vaccinia (7), myxomatosis (8), and influenza (9). Complement-fixing and precipitating antigens are found in the serum during yellow fever infection of monkeys and myxomatosis of rabbits. Although at

first sight it would seem improbable that there are immunological relationships between filterable viruses and malarial parasites, tests for cross reactions were considered desirable because some of these antigens are, perhaps, products of tissue destruction rather than specific components of the virus (6). Sera from monkeys dying of yellow fever and from rabbits dying of myxomatosis were tested for complement fixation with antimalarial monkey sera. Antigens from influenza virus (mouse lung suspension) and lymphocytic chorio-meningitis (guinea pig spleen) were also tested for reaction with antimalarial sera. Immune sera against yellow fever, myxomatosis, lymphocytic choriomeningitis, and influenza were tested for cross reactions with malarial antigens prepared from serum and from parasitized blood. In all cases the results were negative.[1] All of these immune sera gave complement fixation with the corresponding specific antigens.

Attempt to Detect Soluble Malarial Antigen in the Urine of Infected Monkeys

Urine was collected from four monkeys dying of *P. knowlesi* infection. One of these animals had a severe hematuria, the urine being dark reddish brown, and two had milder hematuria. These urines together with specimens from normal monkeys were tested for complement fixation with immune serum. The results were completely negative with amounts of 0.1 cc. of undiluted urine. This negative finding indicates that the malarial antigen does not pass the renal glomeruli.

Production of Complement-Fixing Antibodies by the Injection of the Soluble Malarial Antigen into Monkeys

Citrated serum antigen prepared as described in the section on materials and methods was injected intravenously in amounts of 5 cc. into four normal monkeys. Injections were given on 3 successive days; after an interval of 7 to 10 days three more injections were given on successive days. After this, single injections were

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[1] Several of the monkeys in the malaria animal room became infected with lymphocytic choriomeningitis in February, 1938. The virus was isolated by Dr. T. F. Francis from animals dying of the disease. The sera of some of the survivors fixed complement with choriomeningitis antigen.

given at intervals of 2 to 3 weeks. The total amounts of serum antigen injected into each of the monkeys were 45, 48, 60, and 70 cc., respectively. During the course of the injections animals were bled at intervals of 1 to 2 weeks and the sera tested for complement fixation with serum antigen and with an antigen prepared from red blood cells parasitized with *P. knowlesi*.

· The complement fixation was stronger when the test was done with the antigen prepared from blood than it was with the serum antigen. During the course of immunization two of the monkeys showed maximum complement fixation titers of 1:8 when their sera were tested against the parasitized blood antigen. One monkey showed a maximum titer of 1:16, and the fourth animal a titer of 1:32. These titers are comparable to those of the sera of monkeys with chronic malaria. None of the four animals developed agglutinating antibodies.

The spacing of the injections and the resulting antibody response of one of the monkeys are illustrated in Fig. 2. The antibody titers were determined by complement fixation with an antigen prepared from parasitized blood. 1 week after the first series of three injections there was a slight rise in the titer of antibodies. A specimen of serum taken 1 day after the second series of three injections showed a "negative phase" or decrease in titer. This was followed by a marked rise in the titer of antibodies which reached a maximum after 3 weeks. The titer was maintained near 1:32 by single injections given at intervals of 2 weeks.

The effect of injecting serum antigen intravenously into monkeys with chronic *P. knowlesi* infection is shown in Fig. 3. Blood smears were made daily from these animals and were examined for parasites. Monkey E showed no parasites during the course of the study. The serum of this animal showed a falling complement fixation titer before the injection of serum antigen on Apr. 5. 1 day after the series of three injections there was a definite negative phase, and this was followed by a sharp rise in the titer of complement-fixing antibodies. 2 weeks later the titer had fallen to a level near that which preceded the injection of antigen. Monkey D had parasitic relapses on Mar. 4 and 5 and on Mar. 27 and 28. As has been reported for other experiments in a previous paper (1), relapses are followed by a rise in the titer of complement-fixing antibodies. Monkey D received serum
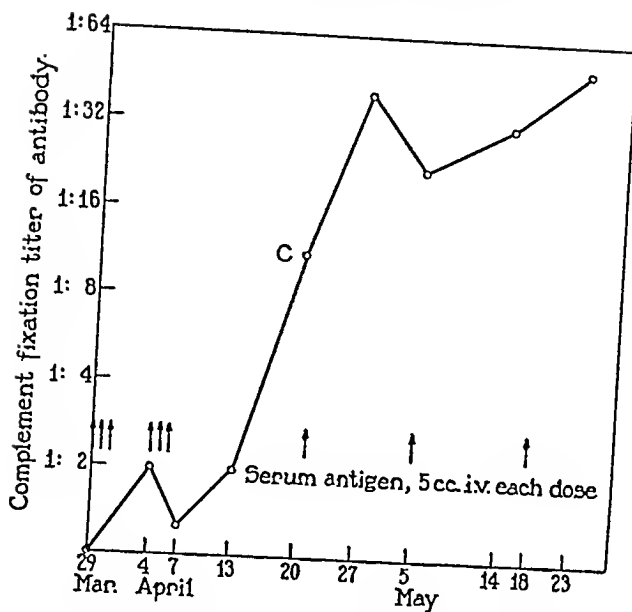
FIG. 2. Production of complement-fixing antibody by injection of malarial antigen.
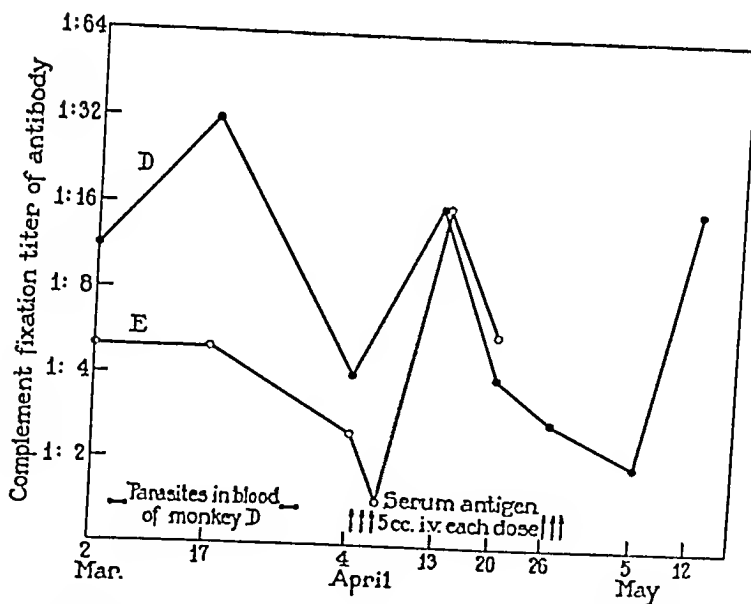
FIG. 3. Stimulation of antibody response by injection of malarial antigen into monkeys with chronic infection.

antigen intravenously on Apr. 5, 6, and 7, and again on Apr. 26, 27, and 28. Since the first series of injections was given shortly after a relapse, the subsequent rise in titer of complement-fixing antibodies

could not be attributed to the injection of antigen. However, no relapses preceded or followed the second series of injections of serum antigen. The resulting rise in titer of complement-fixing antibodies was similar to that produced by a relapse.

Tests for Immunity to Infection

The four normal monkeys which had been injected with soluble malarial antigen were inoculated intraperitoneally with 10,000 living *P. knowlesi* 1 week after a series of injections lasting 6 weeks to 2 months. All four animals became infected after an incubation time which was not appreciably longer than that observed with normal monkeys. One of the animals died with a low parasite count during the early stages of infection and, at autopsy, showed extensive tuberculosis. Two monkeys died with heavily parasitized blood 12 days after inoculation, and the course of the disease in these animals did not differ from that in normal monkeys. The fourth animal, whose serum had a relatively high complement fixation titer of 1:32, developed a very heavy infection but recovered and has remained well for 6 months.

The significance of these results will be considered in the Discussion.

Stability of Serum Antigen

When serum containing the soluble malarial antigen is allowed to stand in the refrigerator, the titer of the antigen gradually falls off over a period of several months. The antigen prepared from parasitized blood also shows a similar lack of stability in a saline solution. Neither antigen is destroyed by heating to 56°C., but a temperature of 70°C. inactivates them. The serum antigen is lost as a result of repeated chemical manipulation. Acids and alkalies destroy both serum antigen and the antigen obtained from parasites. Both may be precipitated by 0.66 saturated ammonium sulfate without appreciable alteration.

Fractionation of Serum Containing the Soluble Malarial Antigen

The serum used in these experiments was obtained from citrated blood by centrifuging down the red cells and defibrinating with calcium chloride solution as described in the section on materials and methods.

Preliminary experiments showed that part of the antigen was carried

down in the globulin fraction precipitated by half-saturated ammonium sulfate, and part remained in the albumin fraction constituting the supernatant. Antigen was precipitated from the globulin fraction by carbon dioxide, but no antigen was precipitated from the albumin fraction by similar treatment with carbon dioxide. Attempts to fractionate the serum antigen by acid precipitation were unsuccessful, the antigen being lost when the pH was brought below 5.5. The studies were, therefore, confined to fractionation by dialysis, ammonium sulfate, and carbon dioxide. During dialysis and fractionation with ammonium sulfate the pH was maintained at all times as near 7.0 as possible.

Experiment 1.—Defibrinated serum giving complement fixation at 1:64 was dialyzed overnight in a cellophane bag. Carbon dioxide was then passed through the dialyzed preparation until no further precipitation occurred. The precipitated globulins were dissolved in saline. This was designated fraction 1*a*. To the supernatant from fraction 1*a* solid ammonium sulfate was added to produce a 0.66 saturated solution. Practically all of the antigen was contained in this precipitate. No antigen could be detected in the supernatant after dialysis. This was discarded. The precipitate produced by 0.66 saturated ammonium sulfate was dissolved in water, the resulting solution dialyzed, and a further portion of the globulins precipitated by carbon dioxide. The redissolved precipitate was designated fraction 2*a*. The supernatant from fraction 2*a* was then fractionated with ammonium sulfate by collecting the precipitates produced successively at 0.33, 0.40, 0.52, and 0.63 of saturation, dissolving in water, and dialyzing the resulting solutions of protein. These fractions were designated 3*a*, 4*a*, 5*a*, and 6*a*, respectively. In all of these fractionation experiments the precipitates were redissolved so as to make a volume of solution approximately half that of the solution from which the precipitate was obtained.

The six fractions just described were tested for complement fixation with the immune monkey serum that was used in detection of the malarial antigen in the whole serum. The results are presented in Table III. The first globulin fraction (1*a*), precipitated by dialysis and carbon dioxide, was very anticomplementary. The second globulin fraction (2*a*), precipitated by carbon dioxide, and the fraction (3*a*), precipitated by 0.33 saturated ammonium sulfate but not precipitated by carbon dioxide, both contained considerable amounts of antigen. The fractions (4*a* and 5*a*), precipitated at 0.40 and 0.52 saturated ammonium sulfate, contained practically no antigen, while the albumin fraction (6*a*) contained the remainder of the antigen in higher concentration than any of the other fractions.

Experiment 2.—The serum giving complement fixation at 1:64 was dialyzed in a cellophane bag as in Experiment 1, but the resulting precipitate of globulin was collected without treatment with carbon dioxide. The globulin precipitate

dissolved in saline was designated fraction 1b. The supernatant from fraction 1b was then fractionated with ammonium sulfate, the precipitates being collected successively from 0.44, 0.54, and 0.66 saturated solution. The last precipitate of albumin was dissolved in water, dialyzed, and tested for complement-fixing antigen without further treatment. This was designated fraction 10b. The fractions precipitated at 0.44 and 0.54 saturated ammonium sulfate were dialyzed separately. Each formed, upon dialysis, a precipitate of globulins, and as these precipitates were found to contain much anticomplementary material, they were

TABLE III

Fractionation of Monkey Serum Containing Soluble Malarial Antigen

Fraction No.	Method of precipitation	Highest dilution giving fixation of complement with immune serum	Highest dilution which was anticomplementary
1a	Dialysis and CO_2	1:64	1:64
2a	0.66 saturated ammonium sulfate and CO_2	1:32	0
3a	0.33 saturated ammonium sulfate	1:16	0
4a	0.40 saturated ammonium sulfate	0	0
5a	0.52 saturated ammonium sulfate	Trace undiluted	0
6a	0.66 saturated ammonium sulfate	1:64	0
1b	Dialysis whole serum	1:64	1:32
2b	CO_2, 0.33 saturated ammonium sulfate, dialysis	1:64	1:8
3b	CO_2 and 0.33 saturated ammonium sulfate	1:8	1:8
4b	CO_2 and 0.50 saturated ammonium sulfate	1:32	1:16
5b	CO_2 and 0.66 saturated ammonium sulfate	1:8	0
6b	0.44, then 0.33 saturated ammonium sulfate	1:32	1:8
7b	0.54, then 0.45 saturated ammonium sulfate	1:4	0
8b	0.54 saturated ammonium sulfate twice	1:4	0
9b	0.54, then 0.66 saturated ammonium sulfate	1:32	0
10b	0.66 saturated ammonium sulfate	1:32	0

For a detailed description of the preparation of the various fractions, see text.
0 = no fixation of complement or no anticomplementary effect.

discarded. Carbon dioxide was then passed through the two dialyzed solutions of proteins (precipitated at 0.44 and 0.54 saturated ammonium sulfate); the two redissolved carbon dioxide precipitates were combined; and the two supernatants were combined.

The carbon dioxide precipitates were refractionated with ammonium sulfate at 0.33, 0.50, and 0.66 of saturation. After being dissolved each of the three fractions was dialyzed. The fraction precipitated at 0.33 saturated ammonium sulfate formed, upon dialysis, a globulin precipitate which was designated frac-

tion 2*b*. The supernatant from fraction 2*b* was designated fraction 3*b*. The other two ammonium sulfate fractions of the carbon dioxide precipitates were designated 4*b* and 5*b*, respectively.

The supernatants remaining after the precipitation with carbon dioxide were also refractionated. Four fractions were successively precipitated with 0.33, 0.45, 0.54, and 0.66 saturated ammonium sulfate, redissolved, and dialyzed to remove ammonium sulfate. These fractions were designated 6*b*, 7*b*, 8*b*, and 9*b*, respectively.

The results of complement fixation tests with the ten fractions just described are presented in Table III. As in the previous experiment the globulin precipitate (1*b*) obtained by dialysis of the whole serum was anticomplementary; the albumin fraction (10*b*) contained much of the antigen. Upon refractionation of those portions of the serum precipitated by 0.44 to 0.54 saturated ammonium sulfate and carbon dioxide, a distribution of antigen between the first globulin fraction (2*b*) and the last fraction (5*b*) was obtained, while the intermediate fractions 3*b* and 4*b* contained little or no antigen. A similar result was obtained by refractionating the serum protein that was precipitated by 0.44 to 0.54 saturated ammonium sulfate but not precipitated by carbon dioxide. Antigen was present in the globulin fraction 6*b* and the albumin fraction 9*b*, but relatively little antigen was found in the fractions precipitated by 0.44 and 0.54 ammonium sulfate (7*b* and 8*b*).

Since antigen precipitable only by 0.66 saturated ammonium sulfate was obtained by refractionation of the globulin precipitates, the results suggest that the antigen found at first in the globulin fraction was merely adsorbed to the protein precipitated by carbon dioxide, or 0.33 saturated ammonium sulfate. However, since a complete separation of antigen from certain of the globulins was not accomplished by refractionation, we cannot exclude the possibility that there is a second antigen having the properties of a globulin which is distinct from the one found in the albumin fraction.

Experiments with High Speed Centrifugation

It was considered possible that the antigen found in the globulin fraction might represent a colloidal suspension of cellular fragments which were carried down mechanically in the first globulin precipi-

tates. In order to test this possibility the following experiment was performed.

Two samples (A and B) of globulins precipitated by carbon dioxide and containing soluble malarial antigen were dialyzed, and the resulting opalescent suspensions were centrifuged at 11,000 R.P.M. for 2 hours. Some protein was thrown down, but all of the antigen remained in the supernatants which were almost entirely clear. The supernatants were then centrifuged at 27,300 R.P.M. for 3 hours in celluloid tubes, each of which contained 6 cc. The vacuum type air-driven centrifuge described by Bauer and Pickels (4) was used. The contents of the tubes were then divided into three levels for preparation A and six levels

TABLE IV

Sedimentation of Soluble Malarial Antigen by High Speed Centrifugation of the Globulin Fraction of Monkey Serum

Preparation tested	Highest dilution giving fixation of complement with immune serum	Highest dilution which was anti-complementary
Original, sample A	1:16	1:4
Upper 2 cc. sample A, centrifuged	1:4	0
Middle 2 cc. " " "	1:8	0
Lower 2 cc. and sediment of sample A, centrifuged	1:32	1:8
Original sample B	1:16	1:8
1st cc. (top) of sample B, centrifuged	Trace	0
2nd cc. of sample B, centrifuged	1:4	0
3rd cc. " " " "	1:6	0
4th cc. " " " "	1:6	0
5th cc. " " " "	1:6	0
6th cc. " " " "	1:16	1:8
Sediment of " " "	1:32	1:16

and sediment for preparation B, using a sampler according to the method of Hughes, Pickels, and Horsfall (10). The centrifuged fractions were then tested for protein, anticomplementary material, and complement-fixing antigen.

The results are presented in Table IV. Most of the anticomplementary material was thrown down by the high speed centrifugation, and the lower third of the liquid and the sediment contained two or three times as much protein as the upper two-thirds A considerable proportion of the antigen remained in the supernatant, and the titer was not reduced much below that of the original material when allowance was made for the anticomplementary properties of the latter.

There was no striking concentration of antigen in the lower levels of the tubes and the sediment, but these portions were more anticomplementary than the original material.

DISCUSSION

The soluble malarial antigen, which is found in considerable quantity in the serum of monkeys during acute infection with *Plasmodium knowlesi*, apparently is concerned, at least in part, in the production of specific complement-fixing antibodies. These antibodies reach a high titer 1 to 2 weeks after the peak of the parasite count. The appearance of complement-fixing antibodies in the serum is accompanied by disappearance of the soluble antigen. The changes in complement fixation titer which occur during acute and chronic *P. knowlesi* infection may be reproduced by intravenous injection into monkeys of parasite-free serum containing the malarial antigen.

Immunization of normal monkeys with the soluble malarial antigen does not give rise to protective antibodies, agglutinating antibodies, or to an efficient active immunity. One of the four *rhesus* monkeys immunized in this way survived after a severe infection, but this result is not considered significant. *Rhesus* monkeys have occasionally survived without treatment after acute infection with *P. knowlesi*. Observations on several hundred monkeys indicate that the mortality of the untreated infection is over 95 per cent. In chronic *P. knowlesi* infection the production of agglutinating and protective antibodies seems to be a slower process than the production of complement-fixing antibodies, and the antigens concerned are apparently distinct from the complement-fixing antigen.

The first results of chemical fractionation of serum containing malarial antigen suggested that two antigens may be present. One of these was precipitated with the albumin fraction. The second was found in a portion of the globulin fraction. The anticomplementary properties of these globulin fractions and the instability of the antigen make it difficult to characterize the substance accurately. Refractionation experiments indicate that at least a part of the antigen found in the globulin fraction was adsorbed to the precipitates. The antigen found in the serum is both chemically and serologically similar to an

antigen extracted from the parasitized red cells. However, the fact that sera from certain *rhesus* monkeys and from human beings infected with *P. knowlesi* give weak or negative reactions with the serum antigen and strong reactions with comparable dilutions of the antigen prepared from parasitized red cells indicates that an additional complement-fixing antigen is present in the parasitized cells.

In its chemical properties the malarial antigen found in the serum of monkeys infected with *P. knowlesi* resembles the antigen found by Hughes (6) in the serum of monkeys dying of yellow fever. The two antigens do not, however, exhibit any serological cross reactions. On the basis of the present evidence, it is impossible to decide whether the malarial antigen concerned in complement fixation is a product of destruction of red cells or a component of the parasite. The fact that the antigen does not produce active immunity to malarial infection is not to be considered evidence of its origin from the red cells rather than the parasites. Certain bacterial antigens, such as the H antigen of the typhoid bacillus, may play an important part in serological reaction but have little to do with protection against infection.

SUMMARY

A soluble malarial antigen which fixes complement with immune serum is found in the serum of monkeys infected with *Plasmodium knowlesi*.

The amount of antigen in the serum is related to the parasite count during the acute phase of the infection. The antigen is not excreted in the urine.

Intravenous injection into normal monkeys of serum containing the antigen stimulates the production of specific complement-fixing antibodies which react with antigens extracted from parasitized cells, as well as with the antigen present in serum obtained during the acute phase of infection.

Monkeys immunized with serum antigen apparently possess very little or no immunity to infection.

The soluble malarial antigen is labile to acids and alkalies, is not destroyed by a temperature of 56°C., and is precipitated, for the most part, in the albumin fraction of the serum by ammonium sulfate.

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EFFECT OF PREGNANCY UPON THE IMMUNITY OF MICE VACCINATED AGAINST ST. LOUIS ENCEPHALITIS VIRUS

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Swiss mice are uniformly susceptible to intranasal or intracerebral injection of St. Louis encephalitis virus. However, when the virus is injected subcutaneously it enters the general circulation but does not multiply in the brain. It produces no lesions in the nerve cells and causes no symptoms of encephalitis. However, such subcutaneous injection produces a strong immunity against subsequent intracerebral injection of the virus (1, 2).

The mechanism by which vaccination renders the brain cells immune is not known. During the course of investigations designed to study this matter, the question of the influence of pregnancy upon acquired immunity arose. Experiments were planned to determine whether pregnancy affected in any way the immunity induced by vaccination with the St. Louis virus.

Materials and Methods

Mice.—All mice employed were of the selected Swiss strain. They are uniformly susceptible to St. Louis encephalitis virus inoculated intracerebrally or intranasally. In all experiments the mated females and their virgin controls were of the same age.

Virus.—The virus used throughout was St. Louis encephalitis virus, strain 3. Virus suspensions were prepared in the following manner: The brain of a mouse prostrate with encephalitis was removed under sterile conditions and ground in a mortar. The emulsion of brain tissue was then diluted with ten times its weight of hormone broth of pH 8.0. After thorough mixing the suspension was centrifuged at 1,000 R.P.M. for 5 minutes and the supernatant made up in serial tenfold dilutions in broth.

Vaccination.—The mice were vaccinated with one subcutaneous injection of 0.5 cc. of a 1:1,000 dilution of virus in broth. On each occasion that the virus was used for vaccination it was titrated by intracerebral injection into virgin fe-

male Swiss mice.   These titrations showed that the amount of virus used for vaccination was approximately 15,000 times the minimal lethal intracerebral dose.   The subcutaneous injection of this quantity of virus never caused encephalitis.

Immunity Tests.—The immunity of the vaccinated mice was tested by intracerebral inoculation of the virus.   All mice inoculated were under light ether anesthesia; each received 0.03 cc. of the virus appropriately diluted in broth. The immunity tests were carried out 2 weeks after vaccination in all experiments except Experiment 7.   On each occasion that the virus was used to test immunity, it was titrated by intracerebral injection into unvaccinated virgin mice.   These titrations showed that each vaccinated mouse tested for immunity received approximately 500 intracerebral M.L.D. of virus.

Mating.—Virgin females $2\frac{1}{2}$ to 3 months old were chosen for mating.   One male was mated to 5 females.   As the females became pregnant, they were removed from the mating box to a separate jar.   The date of delivery of the litter was recorded in each instance.   Unless otherwise noted each litter was kept with its mother throughout the experiment.

Unmated Controls.—In each experiment a number of females were set aside as virgin controls.   These animals were litter mates of those chosen for mating.

Determination of Pregnancy.—In most instances the pregnant mice delivered litters before the end of the experiment.   The usual period of gestation of these mice was about 19 days.   Mice failing to deliver litters 6 weeks after removal from the mating box were considered as non-pregnant.   In one experiment some of the mated mice died a few days before the end of gestation.   In these cases the presence of pregnancy was established by autopsy.

Autopsy Findings.—Practically all of the mice dying following intracerebral resistance tests developed typical symptoms of encephalitis.   In the few doubtful cases the cause of death was proved to be encephalitis by histologic examination and by intracerebral inoculation of the suspected brains into mice.

EXPERIMENTAL

Susceptibility of Unvaccinated Virgin and Pregnant Mice to St. Louis Encephalitis Virus

Virgin mice are very susceptible to intranasally or intracerebrally inoculated St. Louis virus (1, 2).   Numerous titrations of the virus in virgin Swiss mice 1 to 3 months old have shown that intracerebral inoculation of 0.03 cc. of a 1:1,000,000 dilution of virus is practically always fatal.   The same quantity of a 1:10,000,000 dilution of virus kills about two-thirds of the mice injected.   Table I shows that unvaccinated pregnant females exhibit the same degree of susceptibility to the virus as do unvaccinated virgin mice.   It appears that the intracerebral minimal lethal dose of St. Louis virus is the same 'for

the unvaccinated pregnant female as it is for the unvaccinated virgin female Swiss mouse.

Immunity Induced in Virgin Mice by Vaccination with St. Louis Virus

It has already been reported that within one week following vaccination the great majority of virgin Swiss mice become immune to

TABLE I

Susceptibility of Unvaccinated Pregnant and Unvaccinated Virgin Mice to St. Louis Encephalitis Virus

Mice	Dilution of virus injected intracerebrally (0.03 cc. of virus to each mouse)			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Unvaccinated virgin.....................	4/4*	4/4	3/4	0/3
Unvaccinated pregnant†.................	3/3	3/3	2/3	0/3

* 4/4 = 4 of 4 injected mice died of encephalitis.
† These mice delivered litters 1 to 3 days after inoculation of the virus.

TABLE II

Immunity of Unmated Mice Induced by Vaccination with St. Louis Virus

Mice	Dilution of virus injected intracerebrally (0.03 cc. of virus to each mouse)						Immunity induced by vaccination
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Vaccinated virgin.....	1/7*	0/8	1/8	0/8	—	—	10,000 intracerebral M.L.D.
Unvaccinated virgin (controls).........	—	—	4/4	8/8	8/8	2/8	

* 1/7 = 1 of 7 mice injected died of encephalitis.
— = dilution not tested.

10,000 or more intracerebral minimal lethal doses of St. Louis encephalitis virus (1, 2). The immunity remains at this high level about 6 weeks, after which it begins to decline. Table II shows the degree of immunity exhibited by virgin female mice when tested 2 weeks after vaccination.

Influence of Pregnancy upon Immunity Induced by Vaccination

It appears from the data already presented that pregnancy does not render unvaccinated Swiss mice either more or less susceptible to the

St. Louis virus than are unvaccinated virgin mice. The experiments described below were planned to determine whether pregnant mice become immune following vaccination with the virus in the same manner as virgin mice.

Vaccination Late in Pregnancy.—

*Experiment 1.—*In this experiment the immunity induced by vaccination of mice during the last few days of pregnancy was compared with the immunity which follows vaccination of their unmated litter mates.

Twenty-eight female Swiss mice, 2½ months old, were divided into two groups, each consisting of 14 animals. Litter mates were equally distributed between the two groups. One group was mated with males of the same strain, while the other was set aside as unmated controls. All the mated females became pregnant and were not disturbed until 1 to 4 days before the end of the 19th day of the gestation period. At this time they were vaccinated subcutaneously with 0.5 cc. of a 1:1,000 dilution of St. Louis encephalitis virus. That is, the pregnant mice were vaccinated 15 to 19 days after conception. By titration of the virus used for vaccination it was estimated that the vaccinating dose employed was about 15,000 intracerebral M.L.D. Vaccination did not seem to interfere with the normal course of pregnancy; all the pregnant mice delivered normal litters 1 to 3 days after vaccination. The unmated mice were vaccinated at the same time as the pregnant animals, and with the same dose of virus. 2 weeks after vaccination the immunity of both parturient and unmated mice was tested by intracerebral inoculation of about 500 M.L.D. of St. Louis encephalitis virus.

A summary of this experiment is recorded in Table III. The mice vaccinated late in pregnancy failed to develop sufficient immunity to enable them to withstand 500 M.L.D. of the virus. 13 of 14 of them died of encephalitis following the test inoculation of virus, a mortality rate of 93 per cent. In contrast, only 1 of the 14 vaccinated unmated mice died after the intracerebral administration of the virus, a mortality rate of 7 per cent. Calculation of the standard error (3) indicates that this difference in mortality is probably not an accidental one. The result obtained might be expected to occur by chance much less frequently than one time in 100 ($P = < 0.01$).

When the offspring of the parturient vaccinated females became 10 to 13 days old, their immunity against intranasal instillation of St. Louis virus was tested. 92 of 98 of the infant mice inoculated with 100 intranasal M.L.D. of virus died of encephalitis.

Experiment 1 a.—This experiment was carried out in exactly the same manner as Experiment 1. The result was practically identical. 12 of 13 mice vaccinated 1 to 4 days before the end of pregnancy died of encephalitis after the intracerebral immunity test, a mortality rate of 92 per cent.

As before, the great majority of the vaccinated unmated mice were immune; only 2 of 15 (13 per cent) failed to withstand the test dose of virus. Again $P = < 0.01$.

TABLE III

Effect of Pregnancy upon Immunity When Vaccinating Dose Is Administered near End of Pregnancy

Immunity of pregnant mice* following vaccination			Immunity of unmated mice following vaccination	
Vaccinated pregnant mouse No.	Time of vaccination Day of pregnancy	Fate after intracerebral test for immunity (500 M.L.D. of virus)	Vaccinated unmated mouse No.	Fate after intracerebral test for immunity (500 M.L.D. of virus)
1	18th	Died of encephalitis	1	Remained well
2	17th	" " "	2	" " "
3	17th	" " "	3	" "
4	16th	" " "	4	" "
5	18th	" " "	5	" "
6	18th	Remained well	6	" "
7	16th	Died of encephalitis	7	" "
8	18th	" " "	8	" "
9	17th	" " "	9	" "
10	16th	" " "	10	" "
11	16th	" " "	11	" "
12	16th	" " "	12	Died of encephalitis
13	16th	" " "	13	Remained well
14	16th	" " "	14	" "

Mortality of vaccinated pregnant mice = 93 per cent (13/14); mortality of vaccinated unmated mice = 7 per cent (1/14).

* Duration of pregnancy is 19 days in these mice.

The offspring of these vaccinated mothers also showed little evidence of immunity. Of the 80 young mice tested 75 died of encephalitis after receiving 100 intranasal M.L.D. of virus.

Vaccination during Middle Third of Pregnancy.—

The effect of vaccinating pregnant mice during the middle third of the gestation period was next studied as described in Experiment 2.

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The result indicated that the immunity induced by vaccination of mice at this stage of pregnancy was also diminished.

Experiment 2.—This experiment was conducted in the same manner as those already described except that the pregnant mice were vaccinated earlier in the gestation period, that is, 7 to 11 days after the onset of their pregnancy. 2 weeks after vaccination the immunity of the unmated and of the parturient mice was tested by intracerebral inoculation of approximately 500 M.L.D. of virus.

Following this test dose of virus, 9 of 18 vaccinated pregnant mice died of encephalitis, a mortality rate of 50 per cent. As before, the vaccinated unmated mice proved to be immune, only 1 of 15 (7 per cent) died of encephalitis ($P = < 0.01$).

Vaccination Early in Pregnancy.—

Experiment 3.—In this experiment 22 mice were vaccinated during the first 4 days of pregnancy. Their immunity, together with that of vaccinated unmated controls, was tested 2 weeks after vaccination.

The response to vaccination was found to be depressed even very early in the gestation period. 11 of the 22 vaccinated pregnant mice (50 per cent) died of encephalitis after intracerebral inoculation of approximately 500 M.L.D. of virus as compared with only 1 of 14 (7 per cent) of the vaccinated unmated mice ($P = < 0.01$).

Vaccination during the Puerperium.—

The experiments described above indicated that pregnancy inhibits or depresses the immunity which is produced by vaccination. It seemed important to learn whether the power to acquire immunity against the virus was regained after the mice had delivered their young. Accordingly, the response to vaccination carried out at various intervals after the end of pregnancy was studied in Experiments 4, 5, and 6 described below. It was found that the degree of immunity exhibited by the vaccinated puerperal mice during the first 2 weeks after delivery of their young was less than that of vaccinated virgin mice. However, mice vaccinated 7 weeks after the end of pregnancy appeared to withstand the intracerebral inoculation of 500 M.L.D. of the virus as well as did vaccinated virgin mice. Apparently at this time the mice which have reared young have regained at least in part their ability to acquire immunity against the virus. Text-fig. 1 summarizes these results.

Vaccination during the First 2 Days after the End of Pregnancy.—

Experiment 4.—13 Swiss mice were vaccinated against St. Louis virus within the first 48 hours after they had delivered litters. At the same time 12 virgin females were vaccinated. 2 weeks later the immunity of all the mice was tested in the usual way against intracerebrally inoculated St. Louis encephalitis virus.

Seven of the 13 vaccinated puerperal mice (54 per cent) died of encephalitis while only 1 of the 12 vaccinated virgin mice (8 per cent) failed to survive the test virus ($P = < 0.01$).

Vaccination during the 2nd Week after the End of Pregnancy.—

Experiment 5.—This experiment was performed in the same manner as Experiment 4, except that the mice which had borne litters were vaccinated later in the puerperal period; that is, 8 to 12 days after delivery of their young.

Seven of 15 (47 per cent) of the mice vaccinated during the puerperium died of encephalitis following the intracerebral test inoculation of 500 M.L.D. of virus. None of 14 vaccinated unmated mice failed to survive the immunity test ($P = < 0.01$).

Vaccination 7 Weeks after the End of Pregnancy.—

Experiment 6.—In this experiment the mice were vaccinated 7 weeks after they had delivered their litters; that is, 4 weeks after weaning of their young.

Twenty female Swiss mice were mated with 4 males, while 14 of their litter mates were kept as virgin controls. All the mated females delivered litters 19 to 23 days after mating. 50 to 54 days after delivery the young were weaned and removed from their mothers and discarded. About 4 weeks later (more than 7 weeks after delivery) the mice which had reared young and the virgin controls were vaccinated against St. Louis virus in the usual way. 2 weeks later the vaccinated mice were inoculated intracerebrally with about 500 M.L.D. of St. Louis encephalitis virus.

None of the 14 vaccinated virgin controls died of encephalitis and only 2 of the 20 vaccinated mothers (10 per cent). This difference is probably due to chance ($P = 0.21$).

Effect of Pregnancy upon Immunity Established by Vaccination before Mating.—

It seems clear from the data presented that mice vaccinated during pregnancy are in some manner prevented from developing strong immunity against the St. Louis virus. The effect of pregnancy upon immunity already well established before the onset of gestation was

investigated in Experiment 7. The result of Experiment 7 (sum
marized in Table IV) shows that pregnancy brings about a reduction
of the immunity acquired before mating.

Experiment 7.—46 female Swiss mice were vaccinated with St. Louis virus in
the manner previously described. 1 week later 11 of them were chosen at random
and their immunity tested against approximately 500 intracerebral M.L.D. of
virus. All of these mice survived the immunity test and it was therefore assumed
that the remaining 35 vaccinated mice were also immune. 1 week after vaccina-
tion 19 of these latter animals were mated to 5 males of the same strain while 16
were set aside as virgin controls. 16 days later (23 days after vaccination) the

TABLE IV

Effect of Pregnancy on the Immunity Established by Vaccination before Mating

Mice	Time mated	Time of intracere-bral immunity test	Mortality following intra-cerebral immunity test (500 M.L.D. of virus)
Vaccinated virgin........	—	1 wk. after vac-cination	0/11* (0 per cent)
Vaccinated virgin........	—	23 days after vaccination	2/16 (13 per cent)
Vaccinated, then mated (pregnant)............	1 wk. after vac-cination	23 days after vaccination (16 days after mating)	12/18† (67 per cent)

* 0/11 = none of 11 injected mice died of encephalitis.

† Of the 6 survivors in the pregnant group, 3 had been pregnant only 1 or 2
days when given the test virus.

males were removed and the immunity of the mated and virgin vaccinated fe-
males was tested by intracerebral inoculation of approximately 500 M.L.D. of
virus. Of the 19 mated mice, 18 either delivered litters before the end of the
experiment or were found by autopsy to be within 3 or 4 days of term when death
from encephalitis occurred.

Following the inoculation of the test virus, 12 of the 18 pregnant
mice (67 per cent) died of encephalitis as contrasted with only 2 of
16 (13 per cent) of the vaccinated unmated animals ($P = < 0.01$).
It is of interest to note that of the 6 vaccinated pregnant mice which
survived 3 had been pregnant only 1 or 2 days and 2 only 10 days
when the immunity test was done. All the vaccinated pregnant mice

which died of encephalitis had been pregnant 14 to 16 days when they
received the test virus.

DISCUSSION

There are certain indications in the literature that various phys-
iologic factors may influence the reaction of animals to virus infections.
For example, it seems clear that the age of the animal may be im-
portant in this regard. It has been shown by Sabin (4) that old mice
are immune to exceedingly large doses of vesicular stomatitis virus
injected into the leg muscles, while young mice develop a fatal en-
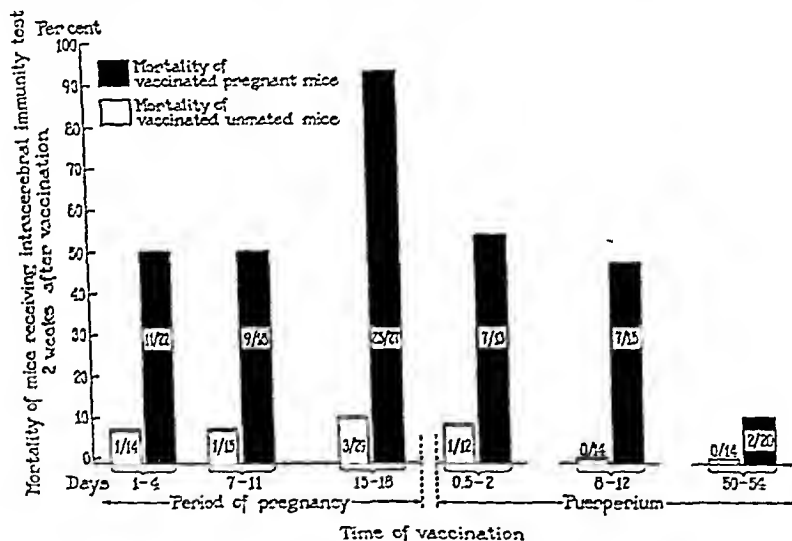
TEXT-FIG. 1. Effect of pregnancy upon immunity produced by vaccination
against St. Louis encephalitis virus.

cephalomyelitis. Sabin stated that in young mice the virus mul-
tiplies at the site of inoculation and invades the sciatic nerve and
spinal cord, while in old mice this invasion of the central nervous
system does not take place.

It has been reported by Sprunt (5) that pregnancy alters the reac-
tion of the tissues of the rabbit to the virus of infectious myxomatosis.
Sprunt reported that following intradermal inoculation of this virus
more extensive lesions occur in the spleens of pregnant animals than
in unmated ones, and that secondary lesions are found uniformly in

the lungs of pregnant animals, while only an occasional lesion is found in the lungs of non-pregnant rabbits. On the other hand the skin of the pregnant animals seems to be less affected by the virus than is the case in the non-pregnant rabbits.

The experiments described in the present communication show that pregnancy does not increase the susceptibility of mice to the St. Louis encephalitis virus, but pregnancy does very definitely diminish the response to vaccination with the virus. This depression of the ability to acquire immunity is most marked late in pregnancy, but it is also demonstrable very early in pregnancy as well as during the first 2 weeks postpartum. However, the immunity response is not permanently reduced by pregnancy. When mice are vaccinated 7 weeks after the end of pregnancy, it is found that their ability to acquire immunity has been regained, at least in part.

It is of interest to note that the offspring of the mice vaccinated during the course of pregnancy are not immune to the virus. Very few of these young mice were able to withstand the administration of 100 intranasal M.L.D. of the virus.

It should be emphasized that pregnancy not only inhibits the development of immunity but that it also adversely affects an already well established immunity. This would suggest that pregnancy exerts its influence not by altering the virus injected as the vaccinating agent but rather that it affects in some manner the tissues which are made immune by vaccination. By what mechanism the state of pregnancy brings about this alteration is not known. Pregnancy is accompanied by very profound physiologic changes in the animal. It seems likely that one or more of these metabolic changes are responsible, although it is impossible at the present time to say which are involved. Experiments designed to study some of these factors, such as the hormones which exert a great influence on the course of pregnancy, are being undertaken.

SUMMARY

1. Virgin and pregnant Swiss mice are equally susceptible to intracerebral inoculation of St. Louis encephalitis virus.

2. Following subcutaneous vaccination with the St. Louis virus, the great majority of virgin Swiss mice become immune to subsequent intracerebral injection of 10,000 M.L.D. of the virus.

3. The majority of mice vaccinated during pregnancy do not become immune to even as little as 500 intracerebral M.L.D. of the virus. The depression of the ability to acquire immunity against the virus is most marked when the vaccination is carried out late in pregnancy, but it is also demonstrable when the mice are vaccinated early in the gestation period and during the first 2 weeks postpartum. At 7 weeks postpartum the response to vaccination is more nearly like that of virgin mice.

4. Pregnancy not only interferes with the development of acquired immunity but it also diminishes a previously established immunity.

5. Offspring of the mice vaccinated during pregnancy are not immune to 100 M.L.D. of virus.

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THE EFFECTS OF ANAPHYLAXIS, AND OF HISTAMINE, UPON THE CORONARY ARTERIES IN THE ISOLATED HEART

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In a previous communication (15), certain manifestations of anaphylaxis in the isolated perfused heart of the guinea pig were described. These were: (*a*) transient increase in the rate and amplitude of contraction, (*b*) delay in auriculoventricular conduction and changes in the form of the ventricular complexes of the electrocardiogram, and (*c*) frequent development of ectopic arrhythmias. In addition to these effects, some of which had already been recorded by the electrocardiograph in the intact animal, an additional effect was reported: a striking *reduction* in the rate of flow through the coronary vessels.[1] The similarity of this reaction to the effect of histamine upon the same preparation was emphasized.

In view of certain differences among various species in the reaction of the coronary arteries to histamine, the authors have proceeded to a study of the anaphylactic reaction in the isolated hearts of cats, in which histamine regularly brings about coronary *dilatation* (8, 2).

Methods

Sensitization to horse serum was accomplished by the intraperitoneal or subcutaneous injection of three doses of 1 cc. each at intervals of 5 to 7 days. Of 14 cats so treated five were tested, from 4 to 10 weeks after the last sensitizing dose, by the intravascular injection of 2 cc. of serum. In one animal no reaction occurred. The remaining four survived prompt reactions characterized by rapid breathing, retching, vomiting and defecation, followed by prostration.

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[1] Similar results have been observed in a small number of experiments with the hearts of sensitized rabbits.

At the time of experiment the sensitized animals and the normal controls were killed instantly by a blow on the head and then their hearts were isolated and perfused with Ringer-Locke solution at 35°C. and a pressure of 75 mm. Hg, and the outflow from the coronary arteries collected and measured, according to the method previously described (15). The antigen, diluted to 1 cc. with warm Ringer-Locke's solution, was injected through the wall of the rubber tubing immediately above the cannula into the stream of the perfusing fluid.

The Results in Normal and Sensitized Animals

The exposure of the isolated heart of the normal cat to horse serum in the way described is not entirely without effect. Within 10 to 15 seconds after the injection the coronary flow suddenly diminishes, returning to its previous rate within 30 seconds. This reaction differs from the true anaphylactic response in at least two important particulars: (a) it is common to normal, sensitized and desensitized hearts alike, and (b) it fails to decrease, but rather tends to increase, in intensity with successive injections.[2] The duration of the effect corresponds more or less closely with the time required for the serum-containing fluid to pass through the heart. A series of 15 tests on four normal hearts produced no other discernible effect. Similar results were observed with desensitized organs.

In the hearts of sensitized cats, on the other hand, after the introduction of 0.01 to 0.1 cc. of serum, the early evanescent coronary constriction is followed by a striking *increase* in coronary flow, beginning about 1 minute after the injection and lasting from 2 to 5 minutes. With subsequent exposures to the antigen this latter effect diminishes in intensity or fails to appear. This conforms with a true anaphylactic reaction. The results of the first injections in ten experiments are summarized in Table I, showing increases of coronary flow of 18 to 48 per cent over the preinjection levels. This reaction is not accompanied by any uniform effect upon cardiac rate, which varies in the same experiments from an increase of 17 per cent to a decrease of 8 per cent of the rate before injection.

[2] The tendency of normal smooth muscle to undergo contraction upon the addition of serum has been observed in other organs suspended in Ringer's solution (12, 13, 4), in perfused blood vessels (3), in the coronary arteries of hearts previously perfused with Ringer's solution (3), and in the pulmonary vessels of normal and sensitized cats (6).
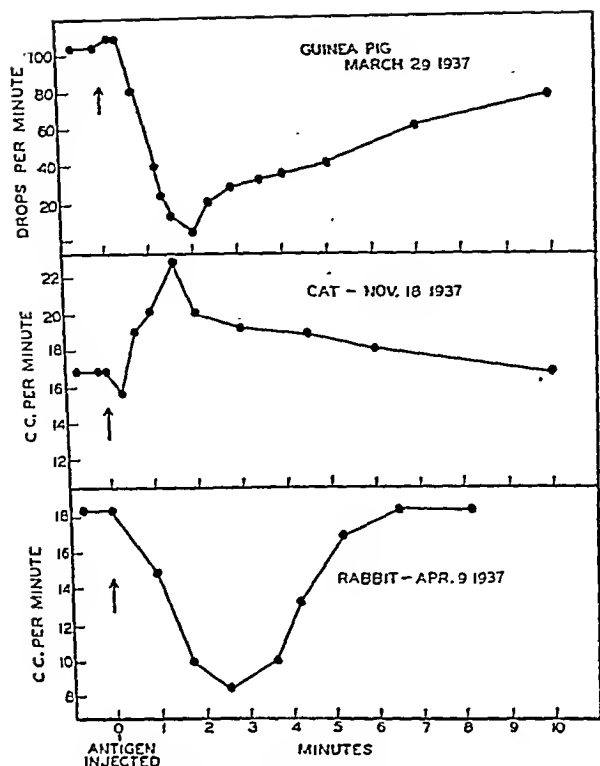
Text.-Fig. 1. The effect of anaphylaxis upon coronary flow in the isolated hearts of the guinea pig, the cat and the rabbit.

TABLE I

Changes in Cardiac Rate and Coronary Flow during Anaphylaxis in the Isolated Perfused Hearts of Cats

Date	Amount of serum injected	Cardiac rate change during reaction	Coronary flow increase during reaction
	cc.	*per cent*	*per cent*
Oct. 14	0.01	−8	35
" 26	0.01	+4	48
Nov. 10	0.01	0	22
" 11*	0.01	−5	30
" 18	0.01	+17	37
" 19*	0.01	0	18
" 29*	0.01	+5	35
Mar. 14	0.01	−7	48
" 15	0.01	−8.5	46
" 22	0.10	4	45

* Animals previously tested by intracardiac injection at 1, 4 and 5 weeks respectively. All three were demonstrably sensitive.

Text-fig. 1 contrasts the effect of anaphylaxis upon the coronary flow in the perfused heart of the cat with that in the heart of the guinea pig and rabbit. In each species these effects are qualitatively identical with the action of histamine upon the same preparation. The results, therefore, further support the view that, in the process of anaphylaxis, histamine or a similar substance is elaborated.

Anaphylaxis in the Hearts of Immune Animals

If repeated doses of horse serum, or other foreign protein, be given to a guinea pig, it may be demonstrated that the animal becomes refractory or "immune" to subsequent injections. These animals will then tolerate the intravenous administration of large amounts of the antigen without symptoms of anaphylaxis. But the isolated uterus of such an immunized guinea pig reacts no less than that of a sensitized animal upon the addition of small amounts of horse serum to its environment (4).

A series of comparable observations was conducted upon the hearts of guinea pigs. The animals were rendered resistant to the effects of horse serum by not less than 12 intraperitoneal injections of 0.1 cc. each—except as noted in Table II—and at intervals of 2 to 3 days. The degree of resistance so engendered was tested by the intravascular injection of serum *in vivo* 1 to 3 days after the last intraperitoneal dose. Under these conditions, four animals bore the intravascular administration of 0.2 cc. or 0.3 cc. of serum without symptoms, and three others 0.6 cc. or 0.8 cc. Three more survived mild reactions after the intravascular injection of 0.8 cc.; one of these tolerated the same dose 3 days later without symptoms. One animal (Dec. 6 *a*) died in typical shock upon the injection of 0.6 cc. of serum 3 days after the last intraperitoneal injection, although the other animal tested on the same day (Dec. 6 *b*) survived without symptoms.

As soon as it became apparent that the intravascular injection would not provoke symptoms, the hearts of seven animals were removed and perfused; two others were isolated 3 and 8 days respectively after the test *in vivo;* four more were tested by the method of perfusion only. In every instance the exposure of the isolated heart to 0.01 cc. of serum in the perfusate caused a typical anaphylactic reaction.

These results suggest anew that the immune guinea pig is protected

by an excess of circulating antibody. But in the heart isolated from such an animal, as in the isolated uterus, the quantity of fixed antibody is sufficient to provoke, upon combination with the appropriate antigen, an anaphylactic reaction indistinguishable from that of the heart of a sensitized animal.

TABLE II

Anaphylactic Reactions in Guinea Pigs Rendered Resistant to Horse Serum by Repeated Injections

Experiment	Number of doses* of horse serum	Intravascular serum *in vivo*		Isolated heart
		Amount	Reaction	Reaction to 0.01 cc. serum
		cc.		
Jan. 25 a	13	0.2	0	++++
" 26 a	13	0.2	0	++++
" 26 b	13	0.3	0	++
" 27 a	14	0.2	0	++++
Dec. 6 b	15	0.6	0	++++
Jan. 24 b	12	0.8	0	++++
†Dec. 7 b	13	0.6	0	Tested Dec. 15
Jan. 25 b	13	0.8	Mild, survived	Not tested
" 28 b	14	0.8	" "	+++
‡ " 25 c	13	0.8	" "	Tested Jan. 28
‡ " 28 a	14	0.8	0	+++
Dec. 6 a	15	0.6	Fatal	Not tested
" 7 a	16	Not tested		++++
† " 15	19	" "		++++
" 8	16	" "		++++
Jan. 24 a	12	" "		++++
" 27 b	14	" "		++++

* 0 1 cc. by intraperitoneal injection at intervals of 2 or 3 days.
† The same animal was used in these two experiments.
‡ The same animal was used in these two experiments.

Double Sensitization

Rosenau and Anderson (11) observed that guinea pigs could be rendered sensitive to three proteins at the same time and that, following a non-fatal reaction to one, such animals were desensitized to that alone. Dale (4) demonstrated multi-sensitization of uterine muscle of the guinea pig to horse serum, sheep serum and egg white, or even to separate serum proteins (Dale and Hartley, 5) but concluded that

desensitization to one antigen was not without effect upon the sensitiveness to the others.

The authors have tested the possibility of double sensitization of the heart in a small group of guinea pigs.   Each animal received, by intraperitoneal injection, 0.1 cc. of horse serum and 0.1 cc. of a 10 per cent solution of egg albumen in Ringer-Locke solution.   4 weeks later, the isolated hearts of four of these guinea pigs reacted to each antigen in small amounts (Table III); after desensitization to one, a typical anaphylactic reaction followed exposure to the other.   A fifth animal died in anaphylactic shock upon the injection of 0.4 cc. serum.   Its heart, removed immediately and perfused, proved completely desensi-

TABLE III

Anaphylaxis in the Isolated, Perfused Hearts of Guinea Pigs Sensitized to Horse Serum and Egg Albumen

Date	First antigen	Reaction	Second antigen	Reaction
Nov. 30, 1937	Serum 0.01 cc.	+++	Albumen 0.1 cc.	++++
Dec. 3, 1937	" " "	++++	" " "	0
Jan. 10, 1938	" " "	++	" " "	++
" 12, 1938	" " "	++	" " "	++++
Nov. 30, 1937	Serum 0.40 cc.*	Fatal	" " "	+++++†
Jan. 13, 1938	Albumen 0.10 cc.	+++	Serum 0.01 cc.	++++

Albumen administered in 1.0 per cent solution.
* Serum injected *in vivo*.
† Perfused heart.

tized to serum but quite sensitive to albumen.   The heart of a sixth reacted on exposure to serum but not to albumen.

A larger series of observations would be required to determine, in this preparation, whether desensitization to one antigen may not exert a quantitative effect upon the sensitivity to another.   But the few results recorded above seem sufficient to demonstrate that the heart of the guinea pig is susceptible to independent sensitization to two different antigens.

Anaphylaxis to a Bacterial Substance

The evidence establishing the occurrence of anaphylaxis induced by bacterial antigens includes reports of the demonstration of the typical

response of the isolated uterus of the sensitized guinea pig upon exposure to appropriate bacterial substances (14, 16). The type-specific polysaccharides derived from the pneumococcus by Heidelberger (9) are not, apparently, capable of bringing about active sensitization in the guinea pig but provoke the characteristic reaction in passively sensitized animals of this species (1) or in the isolated uteri thereof (10).

TABLE IV

Anaphylaxis in the Isolated, Perfused Hearts of Guinea Pigs upon Exposure to Pneumococcus Polysaccharide

Amount of anti-pneumococcus (Type 1) rabbit serum injected intraperitoneally	Elapsed time before experiment	*In vivo* test in intact animal		Isolated heart on perfusion	
		Amount of pneumococcus (Type I) polysaccharide injected intravascularly	Reaction	Amount of polysaccharide I injected	Reaction
cc.	*days*	*mg.*		*mg.*	
0.5	2	0.5	Fatal	Not tested	
2	2	1	"	" "	
3	3	1	"	" "	
1	1	2	"	" "	
2	2	Not tested		2	++++
1	2	" "		1	++++
2	2	" "		1	++++
2	2	" "		0.5	++++
1	2	" "		0.001	++++
1	2	" "		0.0001	++++
1	1	" "		1	0
				2	0
3	3	" "		0.1	0
				0.8	0
				2	0

The authors examined the response of the hearts of sensitized guinea pigs to the polysaccharide derived from the pneumococcus by Felton (7).[3] Attempts to produce active sensitization to this type-specific substance proved entirely unsuccessful. Passive sensitization was accomplished in 12 animals by the intraperitoneal injection of Type I antipneumococcus rabbit serum. The intravenous injection into

[3] The authors are indebted to Dr. Lloyd G. Felton who placed a quantity of this substance at their disposal.

four animals, 24 to 72 hours later, of 0.5 to 2.0 mg. of Type I polysaccharide (Felton), resulted, in each case, in fatal anaphylactic shock. The hearts of six of the remaining eight animals were removed and perfused 2 days after the original administration of antipneumococcus serum. Upon the addition of polysaccharide to the perfusate in amounts as small as 0.0001 mg. there ensued the characteristic anaphylactic response with consequent desensitization (Table IV). This reaction failed to appear in the hearts of the two other animals, isolated 24 hours and 72 hours respectively after the original dose of serum.

SUMMARY

Anaphylaxis in the isolated, perfused hearts of cats has been shown to be accompanied by a considerable, though transient, *increase* in coronary flow. This result is contrasted with that observed in the hearts of guinea pigs and rabbits in which the coronary arteries are constricted during anaphylaxis. Attention is directed to the fact that, in the hearts of these three species, the effects of anaphylaxis and of histamine are qualitatively parallel.

The characteristic anaphylactic response in the isolated hearts of guinea pigs has been evoked: (*a*) in the organs removed from immune animals, (*b*) by each of two antigens (horse serum and egg albumen) under conditions of double sensitization, and (*c*) upon exposure of the hearts of passively sensitized animals to the type-specific polysaccharide of the pneumococcus.

It is evident that, among the effects of anaphylaxis upon smooth muscle in various organs, there must be considered that upon the coronary arteries.

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STUDIES ON THE MECHANISM OF IMMUNITY IN TUBERCULOSIS

THE RÔLE OF EXTRACELLULAR FACTORS AND LOCAL IMMUNITY IN THE FIXATION AND INHIBITION OF GROWTH OF TUBERCLE BACILLI

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PLATES 32 TO 34

The investigations of Rich (1) and his associates have cast profound doubt on the rôle of allergy in immunity to tuberculosis. Their studies intimate that both the fixation of tubercle bacilli at the site of reinfection and the inhibition of their growth may take place in the absence of exaggerated inflammation. Many studies (2) since then have indicated that there is no strict parallel between allergy and immunity. If the fate of the bacilli of reinfection is determined it has been found (3) that their destruction and the inhibition of their growth on intravenous inoculation is most marked in the presence of considerable residual primary lesions and is associated with scant inflammatory response at the site of their focalization. With slight remaining primary tuberculosis the bacilli of reinfection are less effectively inhibited, yet the acute inflammation at the site of localization of the microorganism is much more intense. On introducing melted agar impregnated with tubercle bacilli and trypan blue subcutaneously into normal and highly immunized rabbits (4), it was found that with large doses, the early rush of lymph from the focus of reinfection is so intense that both the bacilli and the trypan blue are swept over to the draining lymph nodes much more rapidly in the sensitized than in the normal animal. However, one cannot conclude from this that in man also the exaggerated inflammation aids rather than hinders the dissemination of the bacilli of reinfection. Rabbits become only moderately sensitized to the tubercle bacillus

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as compared to the exquisite sensitivity that man acquires as a result of a tuberculous infection. It has been shown by Menkin (5) that the fixing capacity of an inflammation is proportional to the injury exerted by the inflammatory agent on the tissues. Therefore the study with a local agar focus was repeated in the guinea pig, the acquired allergic sensitivity of which is more like that of man. It was the purpose of this endeavor to reexamine the immune reactions in the guinea pig and to determine if possible whether allergy plays any rôle in the fixation of the bacilli of reinfection. Incidentally observations have been made that bear on the problem of local immunity.

In the study with the agar focus where the body fluids readily penetrated the agar masses, but into which the cells entered slowly, it was shown that in the acellular agar islands of the normal animal the bacilli multiplied unhindered; in the immune animal, on the other hand, a marked inhibition of their growth was evident in these cell-free areas saturated, as they were, with the immune body fluids. In this paper, based on the use of another procedure, it is felt that more definite evidence of the rôle of humoral bacteriostatic factors in immunity to tuberculosis *in vivo* is presented.

Rôle of Extracellular Factors in the Fixation of Tubercle Bacilli of Reinfection

Methods and Materials

The use of the agar focus has been previously described (4). Guinea pigs were vaccinated with 2 mg. of BCG subcutaneously on the right side of the back near the shoulder. 38 days later, when they exhibited marked sensitivity to tuberculin, these, together with a group of normal guinea pigs, received subcutaneously over the left thigh 5 cc. of a mixture containing 6 per cent agar in saline solution adjusted to pH 7.4, virulent human type tubercle bacilli (P 15 B) and trypan blue. Another series of guinea pigs was given 1 mg. of R 1 tubercle bacilli, a strain of low virulence, intraperitoneally. 53 days later the same animals received 2 mg. of the same culture subcutaneously on the right side of the back near the shoulder. 62 days after this last treatment, the vaccinated, together with a group of normal animals, received 4 cc. of a mixture containing 4.5 per cent agar in saline solution, virulent bovine type tubercle bacilli (Ravenel strain), and trypan blue. In each series the number of bacilli present in the inoculum was determined by culturing unit weights of the agar suspension on Löewenstein's medium supplemented with bone marrow infusion as previously described (6).

At different intervals of time following inoculation a normal and a vaccinated animal were killed. The character of the local and metastatic lesions and their content of trypan blue was noted. The number of living tubercle bacilli present in a unit weight of tissue was determined by culture for the following structures of normal and vaccinated animals: the agar focus with its investing capsule, the superficial inguinal and deep iliac nodes draining this focus, the superficial inguinal nodes on the opposite side, *i.e.* the control inguinal node, and the spleen or liver. The number of colonies cultured from a given tissue was correlated

TABLE I

The Fate of Virulent Tubercle Bacilli and Trypan Blue in an Agar Focus and Their Dissemination in the Body of Normal and R 1 Vaccinated Guinea Pigs

Agar suspension	Time after inoculation	Agar focus				Draining inguinal nodes				Control inguinal nodes		Draining iliac nodes				Liver	
		Normal		Vaccinated		Normal		Vaccinated				Normal		Vaccinated			
		Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Normal	Vaccinated	Colonies	Trypan blue	Colonies	Trypan blue	Normal	Vaccinated
12,400	*days*																
	1	22,200	+++	5500	+++	10	+	0	±	—	0	20	±	0	tr.	0	0
	4	159,000	++	4600	+++	1730	++	10	+	—	0	0	+++	*	+	0	0
	8	29,000	++	48,300	++	8000	+	†	†	—	0	18	++	13	++	40	4
	14	366,000	+	12,000	++	35,600	+	0	tr.	690	0	94,500	+	36	tr.	2100	4
	23	16,600	—	730	—	10,600	—	600	—	—	0	74,900	—	110	—	18,700	7

The intensity of coloration of the agar focus and the draining lymph nodes is graded as follows: tr., trace of blue; ±, faintly blue; +, pale blue; ++, moderately blue; and +++, deep blue.

* Residual regressive tubercle from primary infection; 210 colonies were cultured therefrom.

† This lymph node was enmeshed in the agar focus; 720 colonies were cultured therefrom.

with the histological changes in the tissue immediately adjoining. The sections were stained with Masson's trichrome procedure as modified by Foote (7), by the Ziehl-Neelsen stain for tubercle bacilli and by Mallory's fibrin stain. The fate of the bacilli and the tissue response at the site of inoculation, as well as in the metastatic foci of the normal guinea pigs and those vaccinated with R 1, were compared to the host parasite interactions in the same foci of normal rabbits, and rabbits harboring a primary residual tuberculosis, and inoculated, like the guinea pigs, with comparable amounts of the same culture administered subcutaneously in melted agar and trypan blue. The detailed data for the rabbits have already been reported (4).

Fate of the Bacilli

Since the results obtained from the two guinea pig series were essentially the same, except that with BCG vaccination the immunity exhibited was much less pronounced than that shown by guinea pigs repeatedly treated with the more virulent R 1 culture, the protocols for the former are omitted. In Table I are presented the fate of the bacilli and trypan blue in the normal and R 1 vaccinated guinea pigs.

It will be noted that the inoculum contained a large number of tubercle bacilli, 12,400 colonies being isolated from 10 mg. of the agar suspension. The bacilli in the agar focus of the normal guinea pig multiply almost uninterruptedly up to the 2nd week. By the 4th week, however, there is a marked reduction in the number of tubercle bacilli. Essentially the same observations were made with the agar focus in rabbits (see Table III in previous study (4)). In these animals also the bacilli multiply up to the end of the 2nd week, and a marked reduction in their numbers is found in the 4th week. In the R 1 vaccinated guinea pigs, and in rabbits that harbored a primary infection, the multiplication of the bacilli in the agar focus is markedly inhibited from the beginning. The superficial inguinal node on the side opposite the agar focus, *i.e.* the control inguinal node, was sterile in every case, a fact suggesting that the bacilli cultured from the draining superficial nodes were derived from the bacilli that had invaded these structures from the focus of reinfection, except in the two instances noted in the table.

While the behavior of the bacilli of reinfection at the site of inoculation was the same in rabbits and guinea pigs, the dissemination of the bacilli of reinfection to the draining lymph nodes was fundamentally different. In the former, with a large infecting dose, *viz.* 10,900 organisms per 10 mg. of inoculum, the draining lymph nodes were already invaded within 24 hours, at a time when the draining lymph nodes in the normal animal were sterile. In the guinea pig on the other hand, even with larger numbers of bacilli of reinfection, the draining lymph nodes of the sensitized animal were sterile, although in the normal animal these were already invaded. A similar difference was noted in the spread of trypan blue in reinfected rabbits

and guinea pigs. In the reinfected rabbit the draining lymph nodes contained more trypan blue than those of the normal rabbit. In the guinea pig, however, less trypan blue reached the draining lymph of the vaccinated than of the normal animal. Similar observations were made repeatedly in guinea pigs, both with trypan blue and bacilli, even with still larger reinfecting doses and without agar in the inoculum. The bacilli that rapidly invaded the draining lymph nodes and internal organs in the normal guinea pig underwent massive multiplication in the lymph nodes and liver for the first 2 weeks. In the immune animal on the other hand, few or none were found in the lymph nodes and internal organs 2 weeks after reinfection. In the 4th week, they accumulated but slowly in these draining lymph nodes at a time when large though reduced numbers persisted in the draining lymph nodes, and when uninterrupted multiplication continued in the liver of the normal guinea pig. A similar suppression of multiplication of the bacilli of reinfection that had invaded the draining lymph nodes and internal organs of immunized rabbits has been previously described.

Rôle of Local Immunity

Text-fig. 1 depicts graphically the fate of the bacilli in normal guinea pigs at the portal of entry, in the nearest draining inguinal node, in the more remote draining iliac node, and in a distant internal organ, the liver. The number of tubercle bacilli cultured from the agar focus on the 8th day is indicated but is not integrated in the graph, for this result is obviously atypical, as was evident both from subsequent developments in the agar focus, and also from many other such determinations, both in rabbits and guinea pigs. As noted above multiplication usually continues in the agar focus during the first 2 weeks after inoculation. It is evident that in all these four sites multiplication continues for the first 2 weeks. In the 4th week there is a marked reduction in the number of bacilli at the portal of entry, a much smaller but still definite reduction in the nearest lymph node, a complete cessation of accumulation but no significant diminution in the lymph node more removed from the agar focus and a practically uninterrupted and marked increase of the microorganisms in the distant liver. Essentially similar observations were
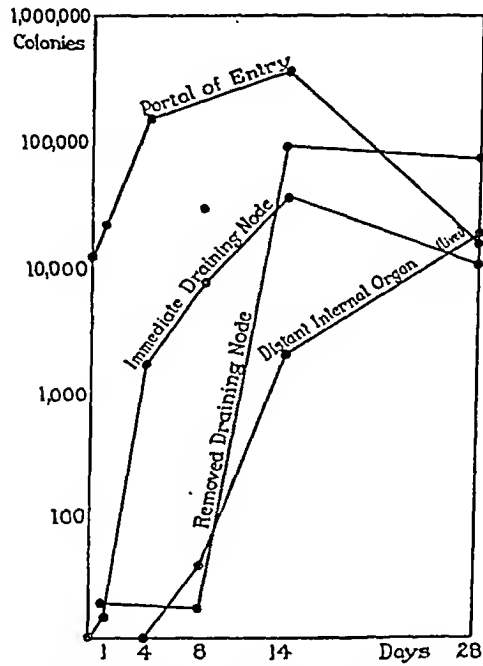
TEXT-FIG. 1. The fate of tubercle bacilli at the portal of entry and at sites more and more remote therefrom in normal guinea pigs.
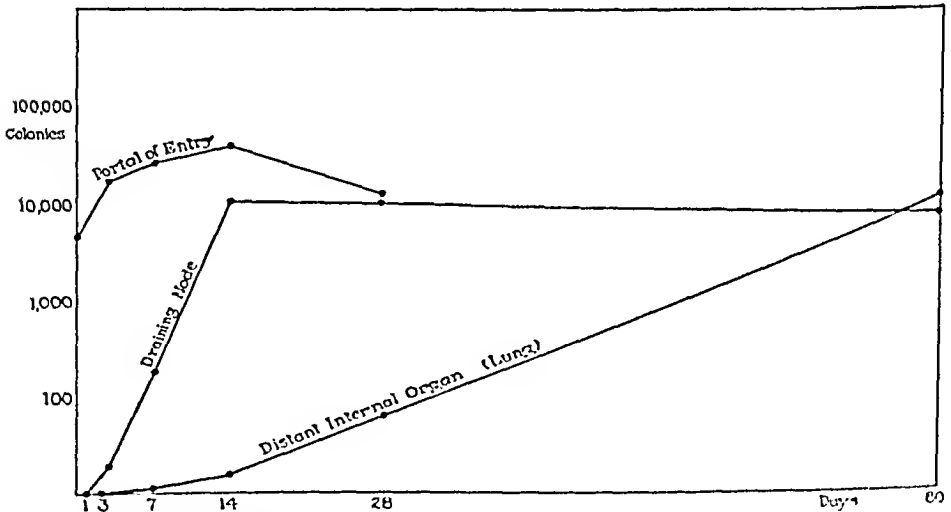
TEXT-FIG. 2. The fate of tubercle bacilli at the portal of entry and at sites more and more remote therefrom in normal rabbits.

made in the rabbit (see Table I in the paper cited (4)), and are illustrated in Text-fig. 2.

It is evident therefore that the fate of the bacilli of primary infection and reinfection at the portal of entry and in the metastatic foci is, with one significant exception considered below, essentially the same in guinea pigs and rabbits. This behavior of the bacilli has already been described for the rabbit in the previous study. Here special attention is directed to the character of the developing immunity in the guinea pig and rabbit primarily infected. It was found that immunity, as determined by the fate of bacilli, appears most rapidly and intensely at the portal of entry of the microorganism, is progressively less effective in the lymph nodes more and more removed from this focus and, at the same time, is altogether ineffective in distant internal organs.

An important difference in the behavior of the two species is observed in the early dissemination of tubercle bacilli and trypan blue from the site of inoculation. With large doses, the bacilli of reinfection and trypan blue reach the draining lymph nodes of the sensitized rabbit more rapidly than they invade the same structures in the normal animal. In the sensitized guinea pig however, even with larger doses, both the bacilli of reinfection and the trypan blue are retarded in their spread to the draining lymph nodes as compared to that in the normal guinea pig. In the succeeding paragraphs an attempt is made to correlate this different behavior of the bacilli in the two species with their respective tissue response.

The Response of the Host

One day after inoculation of agar and tubercle bacilli subcutaneously into normal and vaccinated guinea pigs there is a large accumulation of fluid in the tissues surrounding the agar focus. At the periphery the exudate, situated in proximity to the collagenous fibres, is coagulated, forming a dense fibrinous network (Fig. 3). The outpouring of fluid is much more intense in the vaccinated than in the normal animal. The fibrinous deposits are much more marked in the sensitized animal, extending into the aureolar zone. The lymphatics in the normal animal are patent. In the vaccinated animal they are thrombosed by a fine fibrinous network (Fig. 1). The accumulation of cells about the focus of the vaccinated animal is much greater than that about the normal animal. Many of the polymorphonuclears in the former are necrotic; they are intact in the normal animal. The mononuclears are present in considerable numbers in the

vaccinated; they are scant in the normal guinea pig. The cells lie in a matrix of fibrinous network. This network is more widespread and denser in the vaccinated than in the normal guinea pig. In the normal animal the agar mass itself is broken up into small particles in immediate proximity to the fluid exudate; in the vaccinated this disruption of the agar is much less pronounced. The reaction in the sensitized rabbit differs from that of the sensitized guinea pig in the following respects. The accumulation of fluid about the focus is less pronounced, the polymorphonuclears are less injured and the fibrinous deposit is less marked around the blood vessels. It is coarse, consisting of bulky threads with large spaces between them (Fig. 4), while the fibrinous deposits in the guinea pig, on the other hand, consist of fine fibrillar strands lying close together (Fig. 3). The surrounding lymph vessels in the rabbit are open (Fig. 2), while in the guinea pig they are thrombosed (Fig. 1). The agar mass itself is much more broken up in the rabbit by bulky fibrinous strands than in the sensitized guinea pig.

There was no evidence of growth of the bacilli in any animal 1 day after inoculation. It is noteworthy, however, that while microscopically the bacilli were found in more prominent clumps in the vaccinated guinea pig the tissues of the latter yielded fewer colonies than the agar focus in the normal guinea pig.

In the draining superficial inguinal nodes of the normal guinea pig the marginal sinus was crowded with numerous granulocytes and they pervaded practically the entire node. Fluid distended the intermediate sinuses, and exfoliated macrophages with withdrawn processes were numerous. Many of them contained agar and trypan blue particles. In the draining superficial inguinal node of the vaccinated guinea pig, on the other hand, few granulocytes were present and little fluid was seen in the sinuses. The cells lining the sinuses were sessile, with their processes attached to the walls. They were not exfoliated. They contained little trypan blue, and no agar particles were identified in them. The reverse relationship held in the rabbit. The lymph node draining the agar focus in the normal rabbit was in all respects normal; both the marginal and intermediate sinuses were free of extraneous cells. In the sensitized rabbit on the other hand, the marginal sinus of the draining lymph node was distended with fluid and contained large numbers of polymorphonuclears. Macrophages with engulfed agar particles were seen lying free in the sinuses.

For the first 2 weeks following infection the bacilli in the acellular agar islands of the normal guinea pig were numerous. They appeared as loose masses of deeply acid-fast rods, from the periphery of which numerous bacilli radiated in all directions. They swarmed as dispersed forms uniformly deeply stained, with bulbous ends; branching forms were also seen. They were particularly numerous in proximity to the agar capsule and gradually diminished deep in the agar. They were more prominent in proximity to that portion of the capsule contiguous to the abdominal muscles than to that adjoining the skin. Occasionally deep in the agar were seen colonies which consisted of rounded or oval masses of extremely fine non-acid-fast granules, often arranged about a centrally situated unstained

spherule; from the periphery of this granular body extremely thin, long and short faintly acid-fast rods radiated.

In the immune animal, on the other hand, the bacilli were scanty throughout in the acellular agar, even in immediate proximity to the capsule. They appeared as minute dense clumps of acid-fast, extremely short rods, the individuals of which could not be easily discerned. There were none or few radiating forms about them. The clumps were spotted by numerous black or blue granules. Individual, very short, unevenly stained rods with polar bodies were seen. At times ill defined, weakly acid-fast globules were noted, in the center of which acid-fast granules were found. Rarely minute colonies were encountered, consisting of centrally situated non-acid-fast granules, from the periphery of which a few acid-fast rods sprouted.

In the normal animal the cellular reaction was diffuse, consisting of a large admixture of mononuclears, granulocytes and fibroblasts. No phagocytosis of tubercle bacilli by the mononuclears was noted on the 4th day after inoculation. Later these cells contained large numbers of long, deeply acid-fast rods with bulbous ends, often in the form of packets of parallel rods within the cell.

In the immune animal phagocytosis of tubercle bacilli by mononuclears was noted on the 4th day after reinfection. Well defined tubercles with mature epithelioid cells with well differentiated cytoplasm appeared on the 8th day. Minute foci of caseation were already present. The bacilli were scanty everywhere. They were short and beaded and were found especially in the caseous foci. Both in the normal and in the immune animal the cells lay in a matrix of fibrin. This was particularly conspicuous and extensive in the immunized animal, the cells often lying as if in a basket of intercellular fibrillar substance. By the 2nd week bacilli swarmed within the cells of the normal animal. They were particularly numerous in those central foci of caseation still infiltrated with large numbers of polymorphonuclears. Polymorphonuclears, however, were not limited to these foci but persisted practically throughout the whole lesion. The mononuclears had not yet assumed the shape of mature epithelioid cells; there was as yet no differentiation of the cytoplasm; their processes were still stretched in different directions. The lesion was diffuse and not nodular, invading the surrounding muscle bundles. The latter were often necrotic, apparently choked by the advancing tuberculous process. The enmeshed blood vessels in the necrotic zone were partially thrombosed. Underneath the skin the capsule was less specific in character; fibrous tissue was prominent here.

In the necrotic zone abutting against the agar in the immune animal all the vessels were completely thrombosed. Some of the tubercles were already undergoing regressive changes. The epithelioid cells were rounded and without processes; there was almost no accompaniment by polymorphonuclears. Tubercle bacilli were everywhere scant; short beaded forms were found. The non-specific fibrous tissue formation was much more extensive in the immune animal, and under the skin it was present almost to the exclusion of any specific changes.

In the 4th week the capsule of the normal guinea pig had undergone massive

and diffuse caseation. In those caseous foci where only nuclear debris remained, the bacilli were few, beaded, and short. Caseous foci in an earlier state of development still contained numerous long deeply staining acid-fast organisms. In the vaccinated guinea pig, on the 4th week the same difference from the normal obtained as noted in the 2nd week. Caseation was much less in evidence and fibrous tissue deposits in the capsule were conspicuous.

Thus it is clearly seen that the essential features of the immune response in the rabbit and guinea pig are the same. In both there is suppression of growth of the bacilli of reinfection, both extra- and intracellularly. In both there is a more rapid mobilization of the mononuclears. In both the polymorphonuclears soon disappear from the site of reinfection. In both the phagocytic properties of the mononuclears are greater in the immune than in the normal animal. In both the destruction of the bacilli in the immune animal is associated with a more rapid nodular formation of mature epithelioid tubercles. It is true, however, that nodular tubercle formation is more pronounced in the immune rabbit than in the immune guinea pig; the reaction, even in an immune guinea pig, is still to some extent diffuse.

The chief differences in the response of the two species to reinfection are two. One is the degree of injury the bacilli exert on the tissues of the sensitized animal of these two species. The other is the response of the intercellular substance.

The more highly sensitized tissues of the guinea pig respond with a greater outpouring of fluid, presumably resulting from a greater injury to the vessel walls. The focus becomes shunted off by the clotting of the exudate and the thrombosis of the lymphatics (Fig. 1). The clot is dense and intimate in the guinea pig (Fig. 3); it is loose and coarse in the rabbit (Fig. 4). It is obvious that these extracellular factors form a more effective barrier against the easy dissemination of particulate matter from the site of reinfection in the guinea pig than in the rabbit, where the fibrinous deposits are less pronounced, more porous, and where the draining lymphatics remain open (Fig. 2). It is noteworthy that not only are tubercle bacilli and trypan blue retarded in their passage from the site of reinfection, but agar particles are also prevented from reaching the draining lymph nodes of the vaccinated guinea pig. In the latter they were never found. In

the rabbit, however, the agar is carried to draining lymph nodes of both normal and immunized rabbits. See Fig. 10 and 11 in the paper cited above (4).

The amount and character of the intercellular substance in the two species appear to be operative in the same direction. The fibrinous network in which the cells are lodged is dense and intimate in the guinea pig and very conspicuous (Fig. 7). It is coarse and loose and of much smaller extent (Fig. 8) in the rabbit. Fibrous tissue formation is very much greater in the guinea pig than in the rabbit.

It is interesting to note in this connection that clotted plasma of normal rabbits differs from clotted plasma of guinea pigs. The individual threads in the clot of a rabbit are coarse, with large spaces between them, forming a large meshed sieve (Fig. 6). The individual threads of the clot of guinea pigs are very fine and closely applied, with minute spaces between them, forming a fine sieve (Fig. 5). Furthermore, the plasma clot of a rabbit is soft and friable, and on centrifugation yields its enmeshed serum with ease. The plasma clot of a guinea pig on the other hand is much firmer and less fragile, and on centrifugation its enmeshed serum separates with greater difficulty.

Further observations that suggest mechanical differences in the character of the respective inflammations in the two species have been made. Normal and tuberculous rabbits and guinea pigs were immunized with (a) formalinized typhoid bacilli, (b) a tuberculoprotein, TPT, (8) and (c) horse serum. When the antibody titre of the serum of these animals had attained a certain level, they were given an intrapleural injection of aleuronat-starch. 1 or 2 days later the resulting exudate, freed from cells, was titrated for its agglutinins or precipitins. At the same time the concentration of these antibodies in the corresponding sera of these animals was determined. The results are recorded in Table II.

It is seen that the concentration of agglutinins in the exudate of both normal and tuberculous rabbits is the same as that of the corresponding sera. In the tuberculous guinea pigs, however, while the antibody content of the exudate is also the same as that of the serum of the same animal, it is relatively higher than that of the exudate in a normal guinea pig, the antibody titre of which is lower

than that of its corresponding serum. Again in tuberculous rabbits the precipitin titre of the exudate is often lower than that of the synchronous concentration in their corresponding sera. In tuberculous guinea pigs, however, these antibodies accumulate in much higher titre in the exudate than in the circulating blood.

In other words not only do more precipitins pass from the blood

TABLE II

Synchronous Concentration of Antibodies

In the Blood Serum and in Inflammatory Exudates of Normal and Tuberculous Rabbits and Guinea Pigs

Rabbits			Guinea pigs			Rabbits			Guinea pigs		
Agglutinins			Agglutinins			Precipitins*			Precipitins†		
Rabbit No., normal or tuberculous	Titre of serum	Titre of exudate	Guinea pig No., normal or tuberculous	Titre of serum	Titre of exudate	Rabbit No., normal or tuberculous	Titre of serum	Titre of exudate	Guinea pig No., normal or tuberculous	Titre of serum	Titre of exudate
									15-8 Normal	320	160
50-1 Normal	1280	1280	66 Normal	500	125	G2-20 Normal	5000	5000	15-9 "	640	320
E 3-9 "	1280	1280	69 "	1000	500	B3-12 "	5000	500	16-4 "	320	640
H 2-3 "	320	320	68 "	500	250	C2-19 "	50,000	5000	16-1 "	160	640
E 3-10 "	320	320	70 Tbc.	500	500	A3-3 Tbc.	60,000	80,000	16-3 "	320	640
2 Tbc.	5120	1280	71 "	250	250	C3-1 "	100,000	70,000	14 Tbc.	160	1280
7 "	1280	1280	36 "	500	500	C3-6 "	90,000	80,000	16 "	320	640
A 3-6 Tbc.	1280	1280							40 "	640	2560

* *Versus* tuberculo-protein.

† *Versus* horse serum.

into the exudate to a tuberculous guinea pig than into the site of inflammation of a tuberculous rabbit, but after they have permeated the vessels of the guinea pig they tend to accumulate there until they attain a higher concentration than that in their corresponding serum. This would suggest the following mechanism. The vessels of a tuberculous guinea pig are more injured by the irritant than the vessels of a tuberculous rabbit; hence more antibodies will exude from the blood of the former. But this will not account for the higher concentration of antibodies in the exudate. It may be, however, that the efferent lymphatics at the site of a local non-specific inflammation

in a tuberculous guinea pig are plugged, just as occurs in response to a specific irritant, whereas these vessels in a tuberculous rabbit remain open. In this case the antibodies will leave the site of inflammation in the rabbit, but will be blocked in the guinea pig.

Rôle of Extracellular Factors in the Inhibition of Growth of Tubercle Bacilli of Reinfection

It was shown in the previous study (4) with the agar focus in rabbits and confirmed in the present investigation in guinea pigs that in the acellular agar islands of the normal animal, into which the body fluids penetrate, the bacilli multiply unhindered, whereas in the immune animal a marked inhibition of their growth was evident in these cell-free areas saturated with the immune body fluids. While the observations are suggestive, their interpretation is open to question. The growth of the bacilli in the agar focus is marked at the periphery in proximity to the cellular infiltration. Deeper within the agar the growth is scanty, even in the normal rabbit or guinea pig. Now it has been noted that in the normal animal of both species the agar is broken up into small particles by the invading exudate. In the immune animal on the other hand these particles are larger. It is conceivable, therefore, that the observed suppression of growth of the bacilli in the immune animal may be due, not to the bacteriostatic properties of its body fluids, but to the less effective penetration of these, due to the fact that in the tuberculous animal the agar is little dispersed and in large aggregates. To answer further questions, it was desirable to set up an experiment in which the cells are entirely and permanently kept out from the site of *in vivo* multiplication of the bacilli, although exposed to body fluids.

After numerous trials the following procedure was adopted. Pure, undyed silk, the threads of which were so woven that the interstices between the fibres formed parallelograms about 42 micra in length and 18 micra in width, was sewn into bags. Into the mouth of these bags the lipped rim of a pyrex glass cannula was fastened; the cannula was constricted at its distal third. This bag with its attached cannula was autoclaved and dried under sterile conditions. Because of the shape of the bag, Elford's technique for the preparation of graded collodion membranes of known porosity was inapplicable (9). They were therefore coated with an arbitrary concentration of Mallinckrodt's parlodion which had been autoclaved and dried with sterile precautions. The concentration of parlodion

varied between 2 and 3 per cent by weight. The solvent was 75 to 80 per cent absolute alcohol and 20 to 25 per cent absolute ether. The dry sterilized bags with their attached cannulas were immersed for several hours to several days in the collodion solvent to expel all air bubbles. They were then submerged in the parlodion solution for a similar period. With painstaking sterile precautions, the bags were removed from the parlodion solution, which was allowed to drain off completely. These were now dried in air for about 6 minutes in a vertical position, as routine. The emersion was repeated several times. The junction between the glass cannula and the silk was covered with a thick layer of collodion. Bags so impregnated proved entirely impervious to cells (Figs. 9, 10, 11 and 12) and readily permeable to body fluids. 3 to 4 per cent of molten agar in saline cooled to 50°C. was mixed with a suspension of virulent bovine tubercle bacilli in trypan blue or India ink. By means of a syringe with a long needle the bags were filled with this mixture up to the level of the glass cannula. The cannula was then sealed in the flame at the point of constriction and, after cooling, was placed in the peritoneal cavity of a normal or tuberculous rabbit. At the same time a weighed portion of the mixture of tubercle bacilli and agar was reserved for culture to determine the number of bacilli present in the inoculum before it was placed in the animal.

In a given experiment the preparation of the bags to be used for a normal and tuberculous animal was as nearly identical in each individual procedure as was possible. In some instances, another bag, prepared at the same time, was filled with sterile salt solution, sealed in the flame and placed in the peritoneal cavity of the normal and the tuberculous rabbit simultaneously with the introduction of the bags containing agar and living tubercle bacilli. At different intervals of time, but most often after 2 weeks sojourn in the peritoneal cavity of the normal and immunized animals, i.e., the time of maximum multiplication of locally injected tubercle bacilli, the bags were removed and opened. A large sample of the solid, unbroken, transparent agar was removed, weighed, and used for culture to determine the fate of the bacilli in the agar within the bags. The portion of agar immediately adjoining this sample, together with the tissue membrane that had formed outside and about the collodion-impregnated silk bag in its sojourn in the peritoneal cavity, was prepared for histological study. In some cases, immediately upon opening the silk bag, portions of the agar within were covered with mineral oil for determination of its pH concentration by the method of Hastings and Sendroy (10). On the occasions when blank salt solution-containing bags were also present in the peritoneal cavity, the protein concentration of the fluid within them was determined by the gravimetric method as given by Peters and Van Slyke (11). At the same time its pH concentration was determined. Table III presents the results obtained in 9 such experiments.

It is clearly seen that in all but a single instance the growth of the bacilli within the bags situated in the peritoneal cavity of normal animals is far greater than that taking place within bags placed in a

tuberculous animal. This is sharply brought out in the column listing the ratio between the number of colonies cultured from the agar after its sojourn in the peritoneal cavity and the number obtained from the original inoculum. It will be noted that the number of bacilli within the bags placed in normal rabbits varied from 1.7 to

TABLE III

Fate of Virulent Bovine Tubercle Bacilli within Collodion-Impregnated Silk Bags Placed in the Peritoneal Cavity of Normal and Tuberculous Rabbits

Length of stay of bags in the peritoneal cavity	Number of colonies in inoculum	Number of colonies in bags of				Ratio between number of colonies in bag and in inoculum of		pH of agar in bags of	
		Normal rabbits		Tuberculous rabbits		Normal rabbits	Tuberculous rabbits	Normal rabbits	Tuberculous rabbits
		Rabbit No.	Colonies in bag	Rabbit No.	Colonies in bag				
days									
13	11,700	1	50,000	A 31-6	13,200	4.3	1.1		
14	5900	30-4	140,000	39-6	0*	23.7	—		
14	4900	4	1,400,000†	31-0	33,000†	285.7	6.7		
14	4900	4	190,000‡	31-0	12,000‡	38.7	2.5		
4	4300	3	2600	31-1	300	0.6	0.07		
5	13,200§ 23,200‖	10	210,000	83	30,000	15.9	1.3	7.40	7.40
14	8350	5	160,000‡	E 3-9	1000‡	19.1	0.1	7.25‡	6.95‡
14	8350	5	33,000†	E 3-9	1000†	4.0	0.1	7.35†	7.05†
13	3100	6	133,000	E 3-10	156,000	42.9	50.3	7.35	7.30
14	1500	17-8	2600	6	300	1.7	0.2	7.28	7.05
14	75,000§ 104,000‖	20-3	2,700,000	FM 2	230,000	36.0	2.2	7.22	7.31

* After treatment with sulfuric acid.
† Bag of about 12 mm. diameter.
‡ Bag of about 5 mm. in diameter.
§ Inoculum placed in bag of normal rabbit.
‖ Inoculum placed in bag of tuberculous animal.

285.7 times the number present in the original inoculum. Within the bags placed in tuberculous animals on the other hand, the increase, with the exception of that of E 3-10 noted above, ranged between 1.1 to a maximum of 6.7 that of the original inoculum. In only one instance of a bag placed in a normal rabbit was there an actual re-

duction in the number cultured, as compared with that of the inoculum. It is interesting that in this case the reduction of the number of bacilli within a similar bag placed simultaneously in a tuberculous animal was ten times greater, and that three additional instances of such reduction were found within bags placed in tuberculous animals. The range of dosage in the inoculum varied greatly in different experiments. Presumably also the thickness of the collodion differed in different sets, although in a given set there was little difference in the thickness of the collodion membrane of the bags placed simultaneously in a given pair of normal and tuberculous animals.

Frequently both in normal and tuberculous rabbits, within the glass cannula attached to the bags, above the level of the agar, a clear, cell-free, protein-containing fluid collected, which did not coagulate after several days at 37°C. Likewise within the bags containing salt solution only, identically prepared and placed simultaneously within the peritoneal cavity of normal and tuberculous rabbits, a similar fluid, containing about 3 per cent protein, which failed to clot, was present. It is plain, therefore, that the body fluid penetrated the collodion-impregnated bags placed both in normal and tuberculous animals, but this fluid in a tuberculous rabbit is definitely bacteriostatic as compared with that penetrating a membrane of similar character in a normal animal.

As can be seen from columns 9 and 10 in Table III the pH of the agar within bags in tuberculous animals is frequently considerably lower than that in bags in normal rabbits. However, in three separate experiments where blank, saline-containing bags were examined, there was no difference in the pH of the fluid in these bags placed in a normal or tuberculous animal. Whether this increased acidity of the agar in these instances is due to the differing behavior of the bacilli within them or to some other cause cannot be stated.

Histological preparations of these bags revealed that the contents of the bags remained absolutely cell-free even after 14 or more days sojourn in the peritoneal cavity (Figs. 13, 15 and 16). There was no fibrinous deposit within the bags. Presumably fibrinogen failed to penetrate the collodion, as the protein-containing fluids that penetrated these sacs failed to clot. As a result, the agar remained as

one solid mass, unbroken by the fluids that seeped into it. It is seen therefore that the objections that could have been raised to the interpretation of the results of the agar focus technique as stated above are met in this procedure. As with the subcutaneous agar focus the growth within the bag is chiefly peripheral, being densest in a narrow zone close to the collodion membrane and rapidly decreasing in the deeper layers (Fig. 13). A similar distribution of the bacilli is seen in older caseous foci of infected tissues as illustrated in a caseous lymph node in Fig. 14.

There is usually considerable correspondence between the number of bacilli cultured from within the bags and their histological appearance. They are numerous and appear as actively growing colonies in the bags situated in normal animals (Fig. 9). They are scanty, poorly growing, and often degenerated within bags that had sojourned within the peritoneal cavities of the tuberculous animals (Fig. 10). The differences between them are essentially those previously described in the subcutaneous agar focus of normal and immunized rabbits and guinea pigs.

Two points however require emphasis. It is clear in this procedure that the bacilli frequently grow *in vivo* by the subdivision of the original clump into extremely fine, barely visible, non-acid-fast granules and thin short rods from the periphery of which non-acid-fast, and acid-fast bacilli bud out in all directions (Figs. 15 and 16). Similar observations have been made, as noted above, in the subcutaneous agar focus.

The tissue membrane that forms about these bags is frequently thicker in the normal (Fig. 11) than in the tuberculous animal (Fig. 12). This is to be associated with the fact that the bacilli gradually grow through the collodion which is always saturated with the body fluids. Since the growth is much more pronounced within the bags in normal animals than in those situated in tuberculous animals, this penetration of the bacilli through the surrounding collodion is much more frequent and extensive about bags in normal animals, and tuberculous changes in the investing membrane surrounding these sacs are necessarily much more frequent and conspicuous than in the tissue membrane investing the collodion-impregnated silk bags remaining in the peritoneal cavities of tuberculous rabbits.

SUMMARY AND DISCUSSION

A comparative analysis of the behavior of bacilli of reinfection in immunized and sensitized rabbits and guinea pigs, together with a consideration of the associated host responses in the two species, reveals that the fate of the bacilli and the immune processes of the host are essentially similar in both. However, the bacilli of reinfection are more effectively fixed at the portal of entry in a sensitized and immunized guinea pig than in a comparable rabbit. This has been correlated with the degree of allergy developed by these two types of animals. It is well known that the tissues of a guinea pig become more highly sensitized to the tubercle bacillus than those of a rabbit. The contact of the microorganism with the tissues of the former exerts far more injury upon them than upon those of the latter. A more abundant exudate forms in the guinea pig, the exuded plasma coagulates and, most significantly, thrombosis of the adjoining lymph vessels (Fig. 1) quickly shunts off the focus of reinfection. It is possible that the thrombokinase released by the injured cells may play a part in the observed phenomena. Experiments are under way testing this conception. In the rabbit, on the other hand, the injury of the tissues by the bacilli of reinfection is much less pronounced, coagulation of the exuded plasma is less conspicuous and, particularly, the adjoining lymph vessels remain open (Fig. 2). It would seem also that the fine sieve formation of the clot in the guinea pig (Figs. 3 and 5) as compared with the coarse sieve arrangement of the fibrinous network of the rabbit (Figs. 4 and 6) would also aid in the fixation of substances at the site of inflammation in the former. It is interesting in this connection that the site of a tuberculin reaction in a guinea pig is firm and indurated, whereas in a rabbit it is soft and boggy.

That mechanical differences in the character of the inflammation of reinfection in the two species rather than specific immune processes are involved in the more effective fixation of the bacilli of reinfection in the guinea pig, is suggested by the fact that entirely unrelated substances, such as trypan blue and agar particles, are more effectively localized at the site of reinfection in the guinea pig than in the rabbit. Furthermore in tuberculous guinea pigs at the site of a

non-specific inflammation, blood precipitins accumulate in much higher titre than at a similar site in a tuberculous rabbit. The precipitin titre of the exudate in a tuberculous guinea pig is several times that of the simultaneous titre of its serum. In a tuberculous rabbit it is often lower than the serum titre. It is obvious that only non-specific characteristics of the inflammation in the two species can be involved. It is clear, therefore, that allergic inflammation in the highly sensitized guinea pig mechanically hinders the spread of tubercle bacilli from the site of reinfection. Since the degree of sensitivity of the infected human being is very much higher than that of the guinea pig, it is logical to expect that the inflammation of reinfection in man will have even greater fixing capacities than that of a guinea pig. However the character of the fibrinous network in man is more like that of a rabbit. The plasma clot of man (Fig. 17) forms a network of coarse fibres with large spaces between them. Therefore the exact position of man in this relation still remains uncertain.

While Rothschild (1) and associates demonstrated that desensitization with tuberculin does not lessen the immunity of vaccinated guinea pigs for a considerable time after reinfection, Willis and Woodruff (12) have found that if the desensitized animals are permitted to die from their reinfection they survive a shorter period, develop more extensive pulmonary disease and harbor larger numbers of viable tubercle bacilli in their internal organs than allergic animals similarly reinfected but not desensitized. It is difficult to state to what extent the results of this treatment with tuberculin are due to the removal of the exaggerated inflammatory responsiveness of the tissues to the tubercle bacillus. It is clear, however, that the administration of this agent interferes to some extent, at least, with the immune process.

The importance of the early dissemination of baccilli from the portal of entry is emphasized by the study of the behavior of the bacilli at this site. It has been shown that both in rabbits and in guinea pigs the bacilli are being effectively destroyed at the portal of entry, at a time when they are multiplying practically without interference in distant internal organs (Text-figs. 1 and 2). It is the extension of the tuberculous process in the metastatic foci, and

not at the site of entry of the microorganism, that is significant for the fate of the animal.

Whether this progressive diminution in the inhibition of the multiplication of the bacilli in sites more and more remote from the portal of entry is an expression of a graded local immunity, which develops most rapidly and intensely at the portal of entry and appears later in more distant foci until, at last, the whole organism is immunized, is not so clearly established from these data. It has been shown in previous studies that the maximum immunity developed by a given organ, such as the lung, is never as effective as that of the liver. Nevertheless it is suggestive that Stewart (13) has shown that tuberculin sensitivity develops earlier and in greater intensity in proximity to the primary tuberculous lesion than at a distance from it. Again, as has been shown in a posthumous publication of Sewall (14), the secondary nodule which results 29 days after reinfection of the skin close to the cutaneous primary lesions is much smaller than that which results from reinfection of a skin site remote from the primary lesion. If the size of the lesions is taken as a measure of immunity, it is plain that the immunity in close proximity to a primary focus is greater than that at a more remote site in the same tissue, even in animals that had harbored tuberculosis for 90 days previous to the reinfection; for these were the conditions of the experiments cited. It is significant in this connection that McMaster and Hudack (15) have demonstrated that antibodies are at first present in higher concentration in the lymph nodes draining the site of introduction of the antigen than in the general circulation.

Numerous attempts have been made to demonstrate *in vitro* bacteriocidal properties of serum derived from animals immunized to tuberculosis, but without success. To cite but one experiment, Römer and Joseph (16) exposed 0.000,000,1 mg. of tubercle bacilli, which was the minimal dose still capable of producing tuberculosis in a guinea pig, to the action of 2 cc. of immune serum of a highly sensitized sheep for 24 hours without reducing their virulence. It has been shown in this study that tubercle bacilli exposed *in vivo* to the body fluids of tuberculous animals within collodion-impregnated silk bags, in the complete absence of cells (Figs. 11 and 13) or any other known bacteriostatic factor, are markedly inhibited

in their growth (Fig. 10) for much more extensive multiplication takes place when tubercle bacilli are exposed under identical conditions to the body fluids of a normal animal (Fig. 9). The gradual disappearance of tubercle bacilli from acellular caseous foci is a parallel observation. On the other hand the inordinate growth and swarming of tubercle bacilli in old cell-free caseous foci undergoing softening indicates that other unknown factors may intervene to overcome this bacteriostatic property of the body fluids in the immune animal. To what extent, if any, the local accumulation of antibodies from the blood at the site of reinfection in highly sensitized animals aids in their local suppression of growth is uncertain.

The complete absence of cells from these *in vivo* growth sites of the tubercle bacillus has afforded an opportunity to confirm the studies of Kahn (17). Under certain conditions, the tubercle bacillus does not grow by fission only, but by preliminary subdivision into fine non-acid-fast granules from which both non-acid-fast and acid-fast bacilli sprout (Figs. 15 and 16). It is perhaps this non-acid-fast state of the bacilli which may explain the incongruity often observed between cultural and inoculation methods on the one hand, and histological procedures on the other, in the demonstration of tubercle bacilli.

CONCLUSIONS

1. The fate of bacilli of reinfection at the portal of entry and in metastatic foci, and also the associated host responses, are essentially similar in rabbits and guinea pigs.

2. However, in the guinea pig tubercle bacilli of reinfection are more effectively fixed at the portal of entry than in the rabbit.

3. The guinea pig fixes at the site of reinfection unrelated substances, such as trypan blue and agar particles, more effectively than the rabbit.

4. At the site of a local non-specific inflammation precipitins from the circulating blood accumulate in higher concentration in tuberculous guinea pigs than in tuberculous rabbits.

5. These differing fixing capacities of the two species are associated with differences of extracellular character in the inflammation resulting from reinfection. (*a*) In the guinea pig, whose tissues are

highly sensitized and greatly injured by the tubercle bacillus, the lymphatics adjoining the site of reinfection become thrombosed. In the rabbit whose tissues are moderately sensitized and less injured by the tubercle bacillus the corresponding lymphatics remain open. (b) In the guinea pig the fibrinous network at the site of inflammation forms a fine sieve-like structure. In the rabbit this network forms a coarse sieve-like barrier.

6. In rabbits and guinea pigs primarily infected, the destruction of tubercle bacilli takes place first and most extensively at the portal of entry. At this time they are less effectively destroyed in the nearest metastatic foci. Simultaneously they are still growing without hinderance in such foci in remote internal organs.

7. The cell-free body fluids of normal animals support the growth of tubercle bacilli *in vivo*. The body fluids of tuberculous animals under the same conditions are bacteriostatic for this microorganism.

8. Tubercle bacilli often multiply by preliminary subdivision into non-acid-fast granules, from which the acid-fast rods sprout. This confirms the work of Kahn.

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EXPLANATION OF PLATES

All sections were prepared from tissues stained with Masson's trichrome procedure except those depicted in Figs. 9, 10, 13, 14, 15 and 16 which were stained by the Ziehl-Neelsen method, and counterstained with hematoxylin. The magnifications given are approximate.

PLATE 32

FIG. 1. Thrombosed lymphatic at the site of inoculation of a BCG vaccinated guinea pig, 1 day after reinfection. The lymph node draining this focus was sterile. × 700.

FIG. 2. Open lymphatic at the site of inoculation of a tuberculous rabbit, 1 day after reinfection. A few coarse fibrin threads are present within the lumen. The lymph node draining this focus yielded 16 colonies. × 700.

FIG. 3. The fibrinous exudate at the site of reinfection in the sensitized guinea pig shown in Fig. 1. The fibrin threads are fine and the spaces between them are small, forming a dense sieve-like structure. × 700.

FIG. 4. The fibrinous exudate at the site of reinfection in the sensitized rabbit shown in Fig. 2. The fibrin threads are bulky and the spaces between them are large, forming a coarse sieve-like structure. × 700.
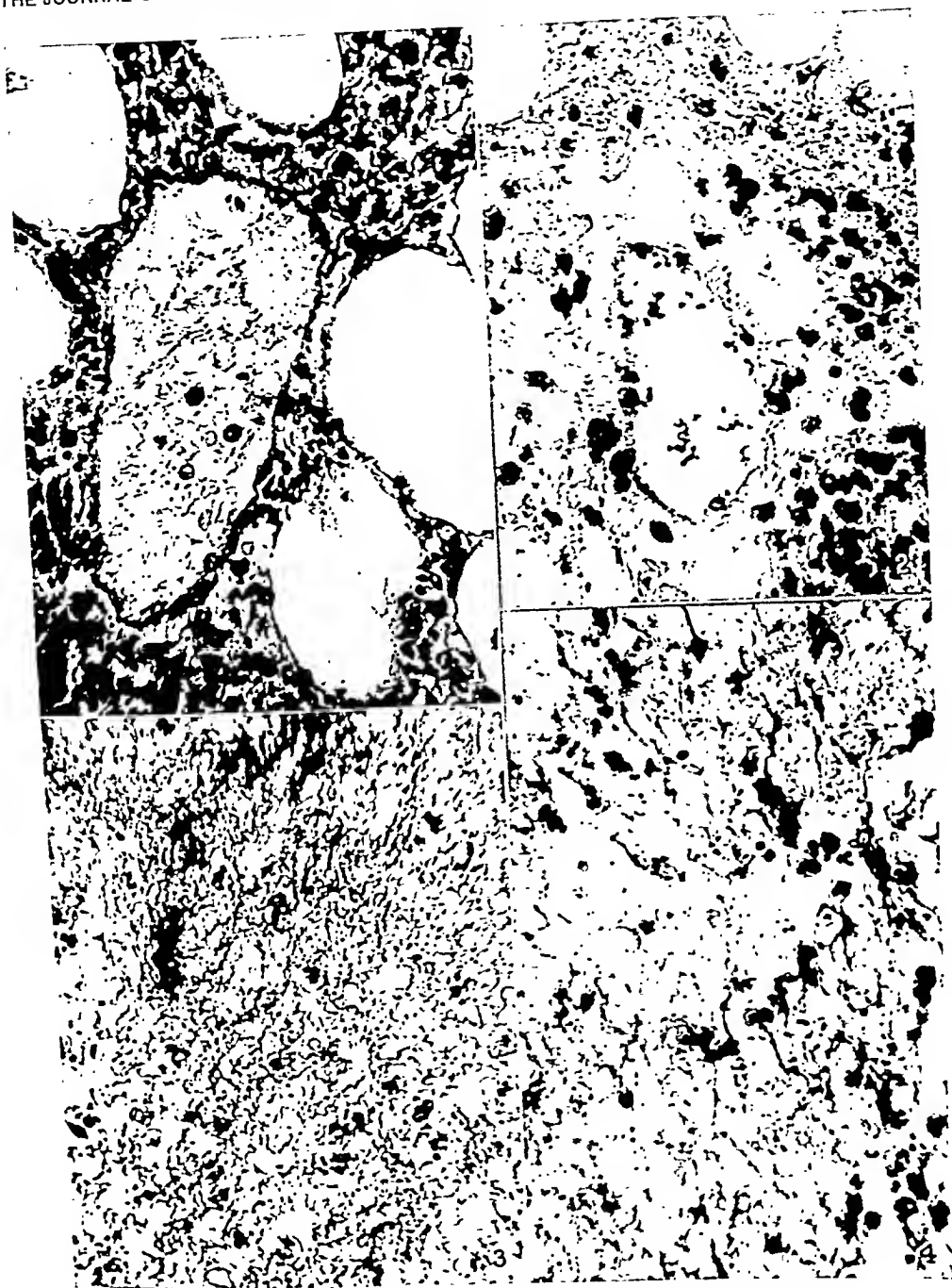
PLATE 32

PLATE 33

FIG. 5. Clotted plasma of a normal guinea pig. The closely lying fine fibrin threads form a dense network. × 700.

FIG. 6. Clotted plasma of a normal rabbit. The bulky dispersed fibrin threads form a coarse network. × 700.

FIG. 7. The capsule surrounding the agar focus in vaccinated guinea pig 18-2, 2 weeks after reinfection. Fibrillar intercellular substance is conspicuous. × 700.

FIG. 8. The capsule surrounding the agar focus in tuberculous rabbit 14-3, 2 weeks after reinfection. Intercellular substance is very scant. × 700.
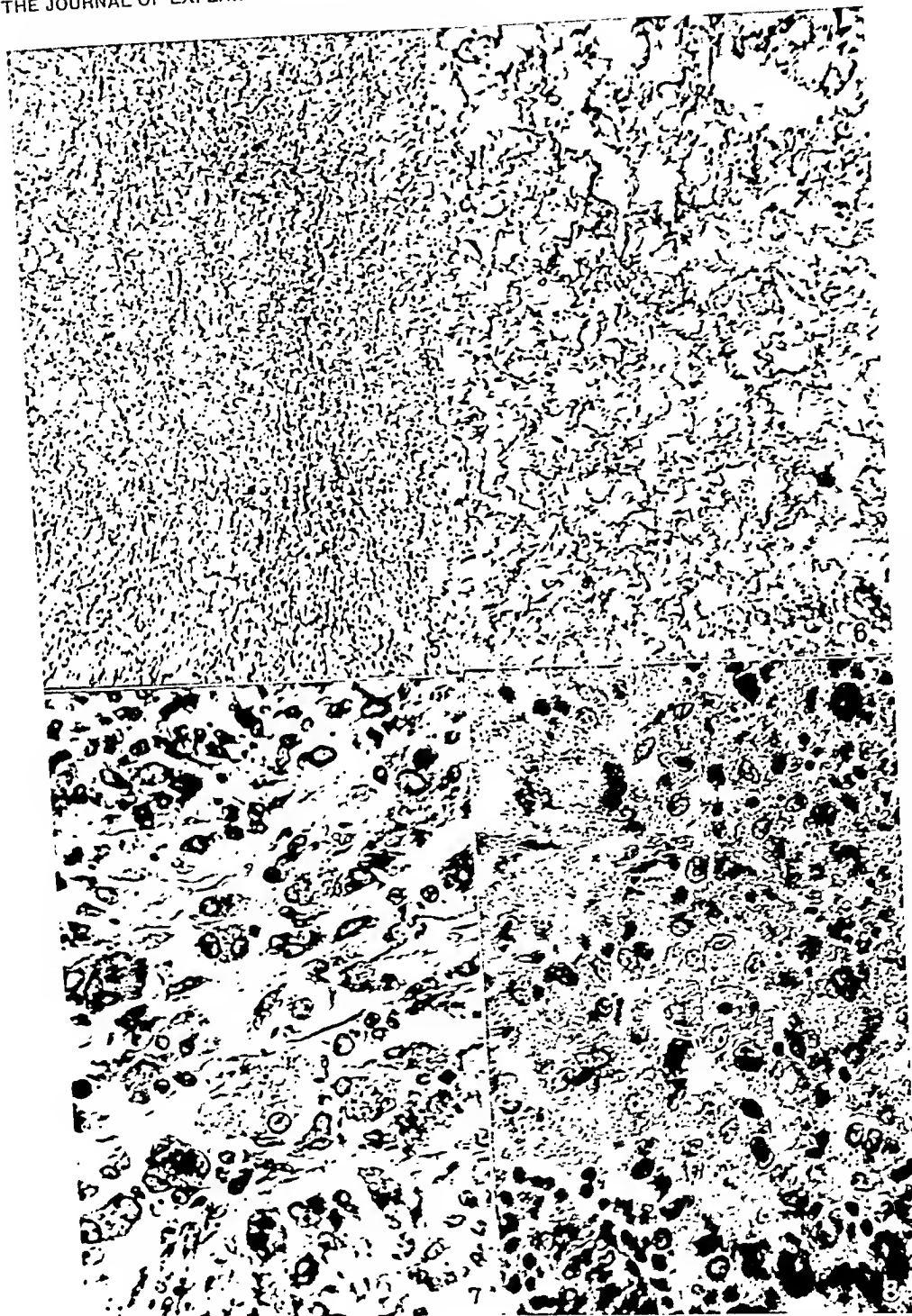
PLATE 33

PLATE 34

FIG. 9. Tubercle bacilli growing in small bag placed in normal rabbit 5, after 2 weeks stay in the peritoneal cavity. 160,000 colonies were recovered from 10 mg. of agar within this bag. Ratio between number of colonies in bag and that in original inoculum, 19.1. × 700.

FIG. 10. Tubercle bacilli growing in small bag placed in tuberculous rabbit E 3-9 simultaneously with that placed in normal rabbit 5, shown in Fig. 9, after 2 weeks stay in the peritoneal cavity. 1000 colonies were recovered. Ratio between number of colonies in bag and that in original inoculum, 0.1. × 700.

FIG. 11. Thick membrane investing collodion-impregnated bag placed in normal rabbit 4, after 14 days stay in the peritoneal cavity. The leukocytes come up to the collodion but do not pass through. Ratio between number of colonies recovered from agar within this bag and that from original inoculum, 285.7. × 160.

FIG. 12. Thin membrane investing the collodion-impregnated bag placed in tuberculous rabbit 31-0 simultaneously with bag placed in normal rabbit 4 shown in Fig. 11, after 14 days stay in the peritoneal cavity. From above downwards the following structures are encountered: the tissue membrane investing the bag, the collodion on the outside of the silk threads, the silk threads cut across, the inner layer of collodion, and the acellular agar within the bag. Ratio between number of colonies recovered from agar within this bag and that from original inoculum, 6.7. × 160.

FIG. 13. Growth of tubercle bacilli within collodion-impregnated silk bag placed in normal guinea pig 15-2, after 4 weeks stay in the peritoneal cavity. Cells are completely absent. The colonies of tubercle bacilli are limited chiefly to a narrow zone on the periphery of the unbroken agar adjoining the sac, which was below the figure. The growth is scant in the deeper regions. × 160.

FIG. 14. The periphery of a caseous lymph node of a rabbit injected with bovine tubercle bacilli. The microorganisms are present in large numbers near the edge of the node close to the fibrous capsule, which is situated to the right of this figure. The bacilli are fewer in the deeper layers of the node. × 700.

FIG. 15. Colony of tubercle bacilli growing in bag placed in normal rabbit 4 after 14 days stay in the peritoneal cavity. The light particles in the center are non-acid-fast granules. The dark rods are short acid-fast bacilli sprouting from the periphery of the granular body. × 1500.

FIG. 16. Colonies of tubercle bacilli growing in same bag as in Fig. 15. Long, deeply acid-fast, dark rods are seen radiating from a centrally situated light, non-acid-fast, faintly granular body. × 1500.

FIG. 17. Clotted human plasma. The network formed by the fibrin resembles that of a rabbit shown in Fig. 6. × 1500.

PLATE 34
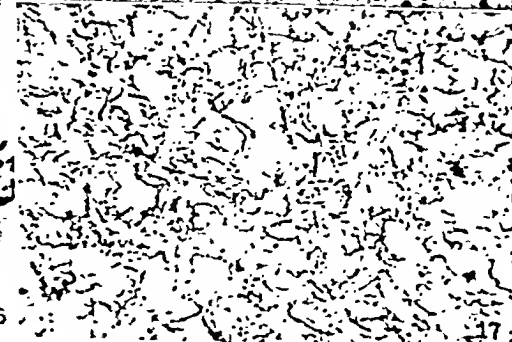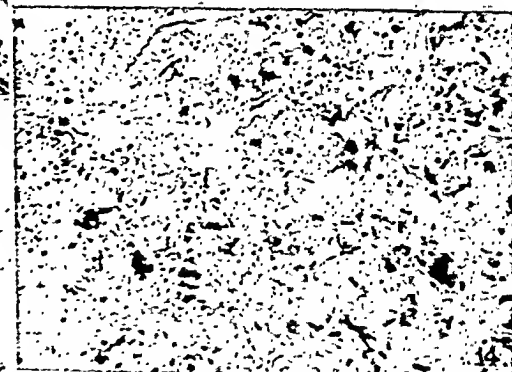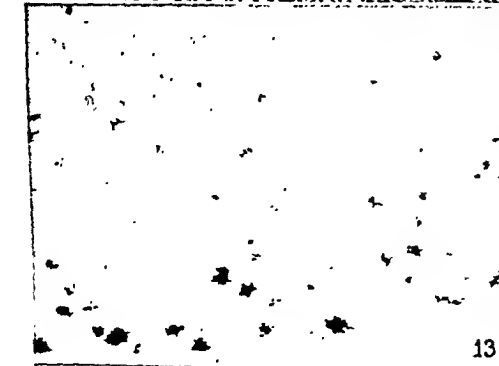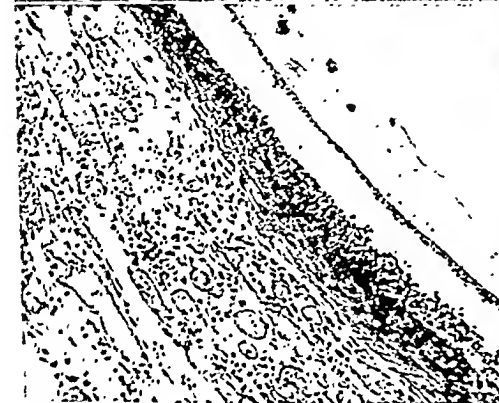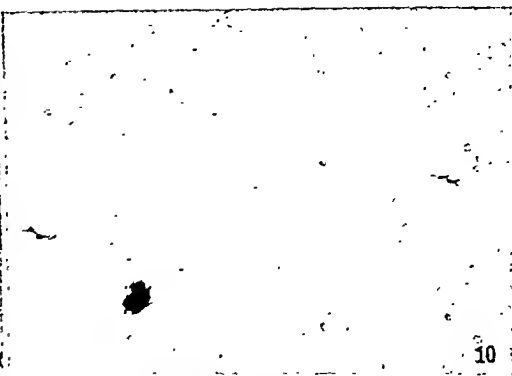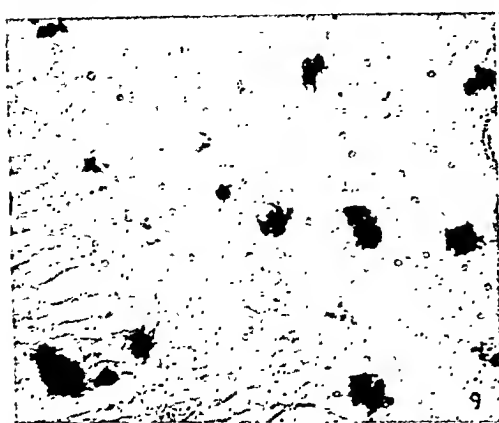
STUDIES ON THE MECHANISM OF IMMUNITY IN TUBERCULOSIS

THE MOBILIZATION OF MONONUCLEAR PHAGOCYTES IN NORMAL AND IMMUNIZED ANIMALS AND THEIR RELATIVE CAPACITIES FOR DIVISION AND PHAGOCYTOSIS

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PLATES 35 AND 36

It is generally recognized that the lesion of reinfection in tuberculosis differs from that of the primary infection by the acceleration and intensification of the immediate inflammatory reaction, by the quickened formation of nodule and tubercle and by their abortive nature. In previous studies (1) it was concluded that the most significant factor in the mechanism of immunity to tuberculosis is the rapid mobilization of mononuclear phagocytes with an increased physiological capacity to destroy or inhibit the growth of tubercle bacilli; the more rapid formation of epithelioid tubercles and their ready resolution were attributed to these factors. Recently Dienes and Mallory (2) have also shown that normal guinea pigs respond with an exudation of polymorphonuclears to the introduction of tubercle bacilli, but, following tuberculous infection, and synchronously with the development of hypersensitivity to tuberculin, the reinjection of this microorganism elicits a predominantly mononuclear reaction. These investigators therefore consider the quickened mobilization of these phagocytes as the result of the allergic state.

The chief present endeavor was to elucidate the mechanism of this accelerated mobilization of mononuclears which characterizes the response to reinfection of the tuberculous or allergic animal.

The old studies of Müller (3), the more extended observations of Opie (4), and the recent investigations of Weiss (5) have indicated

* With the technical assistance of Mr. Peter Zappasodi.

that the proteolytic enzyme of polymorphonuclears, leukoprotease, is active in a neutral or slightly alkaline medium. Lymphoprotease, the enzyme derived from mononuclear phagocytes, is active in an acid medium. Menkin (6) correlated the leukocytic formula and the pH of the medium during the course of an acute inflammation. He found that the polymorphonuclears persist in an exudate the pH of which is above 7.0. When the pH level falls to 6.9 or 6.8 the mononuclears predominate. Therefore it seemed pertinent to determine whether the more ready mobilization of the mononuclear phagocytes in the immune animal may not be associated with a more acid reaction at the site of reinfection than at the site of a primary infection. Furthermore, since it was found that in the acellular areas of a subcutaneous agar focus containing tubercle bacilli, the microorganism fails to grow in the body fluids of the immune animal (1, 1936), it was thought possible that a local tissue acidosis (7) might conceivably develop at the site of reinfection which would account for the bacteriostasis. This seemed the more plausible since Dernby and Avery (8) found that acidity *in vitro* was bacteriostatic for pneumococci, and since, furthermore, as had been determined by Lord and Nye (9), a pH lower than 6.8 will kill pneumococci *in vitro*. Therefore the following experiments were performed.

The pH at the Site of Reinfection and the Fate of the Bacilli

6 per cent agar in saline, adjusted to pH 7.4, was melted, cooled to 50°C., mixed with a suspension of virulent bovine tubercle bacilli and injected subcutaneously into normal and tuberculous rabbits. Normal guinea pigs and guinea pigs vaccinated with the attenuated tubercle bacillus, R 1, received a similar mixture of agar and tubercle bacilli in one flank and the same amount of melted agar without tubercle bacilli in the opposite flank. At different intervals of time following the injection, the rabbits were lightly anesthetized with ether and the guinea pigs with sodium amytal. The skin over the agar focus was quickly reflected and, with dry scissors, snips of agar were removed and immediately submerged in liquid petrolatum. The pH of this agar was determined by the method of Hastings and Sendroy (10).

It can be seen from Table I that up to the 2nd week following inoculation, the pH of a subcutaneous agar focus containing virulent tubercle bacilli was distinctly lower in the tuberculous rabbit or the vaccinated guinea pig than in the corresponding normal control.

That this greater acidity at the site of reinfection than at the site of a primary inoculation is not to be associated with the varying behavior of the tubercle bacilli in the normal and immunized animals is suggested by the observation that even in the absence of tubercle bacilli the pH of an agar focus in vaccinated guinea pigs was lower than in normal animals. Similar differences were noted in the pH of the contents of collodion-impregnated silk bags placed in the peritoneal cavities of normal and tuberculous rabbits (11).

Although the hydrogen ion concentration was definitely lower at the site of reinfection than at the site of a primary inoculation, the

TABLE I

The pH of a Subcutaneous Agar Focus with and without Tubercle Bacilli in Normal and Immunized Rabbits and Guinea Pigs

Interval after injection of agar	Agar focus with tubercle bacilli in rabbits		Agar focus with tubercle bacilli in guinea pigs		Agar focus without tubercle bacilli in guinea pigs	
	Normal	Tuberculous	Normal	Vaccinated	Normal	Vaccinated
1 day........................	7.55	7.30	7.40	7.25	7.15	6.85
3 days.......................	—	—	7.20	6.85	6.95	6.70
1 wk.........................	7.35	7.05	7.00	6.80	7.15	6.70
2 wks........................	7.20	7.10	6.90	7.05	6.85	7.00

difference was only about 0.2 of a pH. It does not seem likely that this difference can play a significant rôle in the inhibition of growth of tubercle bacilli in the immunized animals, especially since Long (12) found no difference whatsoever in the growth energy of tubercle bacilli between pH 6.4 and 7.8; and even the pneumococcus is injured by a low pH only in the absence of body fluids, but, in their presence, it survives and multiplies at a pH of 5.5 (13).

The pH at the Site of Inflammation and the Mobilization of Mononuclears

While the greater acidity at the site of reinfection does not explain the inhibition of growth of tubercle bacilli in the immunized animals, does it account for the more rapid appearance of mononuclear phagocytes in these foci? If the pH of the medium in an acute inflammation is a significant factor in the mobilization of the cells it should follow from the considerations noted above that sensitized guinea pigs would respond to a non-specific irritant with an exudate containing more

mononuclears than normal animals, since it was found that even in the absence of tubercle bacilli the site of a local inflammation in a vaccinated guinea pig is more acid than a similar site in a normal animal.

TABLE II

The pH of the Exudate and Its Leukocytic Formula after an Intrapleural Injection of Aleuronat-Starch, together with the Simultaneous Leukocytic Formula of the Blood in Normal and Sensitized Guinea Pigs

Interval after injection	Guinea pig No.		pH of exudate		Leukocytic formula of exudate				Simultaneous leukocytic formula of the blood			
					Normal		Sensitized		Normal		Sensitized	
	Normal	Sensitized	Normal	Sensitized	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes
1 day	18-6 L	18-6 B*	7.15	6.65	96.4	3.6	79.0	21.0	69.0	31.0	55.6	44.4
" "	32-2	1*	7.15	6.95	93.0	7.0	43.5	56.5	63.0	37.0	78.0	22.0
" "	A	4*	7.15	6.95	96.0	4.0	68.5	31.5	85.0	15.0	51.0	49.0
" "	19-2	30-0*	7.30	7.00	88.0	12.0	87.5	12.5	30.0	70.0	63.0	37.0
" "	10-2	10-3†	7.20	7.10	91.2	8.8	86.7	13.3	55.5	44.5	74.0	26.0
" "	11-0	10-6†	7.10	6.75	86.8	13.2	78.1	21.9	60.0	40.0	69.0	31.0
" "	49	40†	7.30	7.10	87.5	12.5	80.0	20.0				
" "		41†		7.10			67.5	32.5				
Average.....			7.20	6.95	91.2	8.8	73.8	26.1	60.4	39.6	65.1	34.9
2 days	49	40†	7.25	7.35	79.0	21.0	72.3	27.7				
" "		41†		7.15			38.5	61.5				
3 "	10-2	10-3*	7.35	7.20	88.5	11.5	78.5	21.5	30.0	70.0	39.3	60.7
" "	11-0	10-6*	7.15	6.70	71.3	28.7	66.2	33.8	41.0	59.0	34.0	66.0

* Tuberculous guinea pigs.
† R 1 vaccinated guinea pigs.

For this purpose a group of normal and sensitized guinea pigs were injected intrapleurally with a mixture containing 5 per cent aleuronat and 3 per cent starch. At different intervals thereafter the exudate was withdrawn and its pH was immediately determined by the method of Hastings and Sendroy. Simultaneously smears were made of this exudate as well as of the venous blood, in order to determine whether there was any correlation between the pH of the exudate and its leukocytic formula, and also, whether the cells in the exudate were a reflection of those circulating in the blood. The smears were prepared with Wright's stain according to Osgood's modification (14). At least 200 cells were counted for differentiating the cells.

It can be seen from Table II that actively tuberculous and R 1 vaccinated guinea pigs mobilized mononuclears much more quickly than normal animals, even in response to a non-specific irritant. Thus, in 7 normal animals, 24 hours after injection, the non-granulocytes constituted an average of 8.8 per cent of the mobilized cells. Under the same conditions these cells constituted 26.1 per cent in sensitized animals. Furthermore the lead that the mononuclears attained in the exudate of the sensitized animal on the 1st day after the onset of the inflammation was maintained on the 3rd day, when the mononuclears were still present in greater percentage in the exudate of the allergic animal. As can be seen from the last four columns of this table the cell constituents of the circulating blood bore no constant relation to the exudate cells. The latter, therefore, were not solely determined by the cells circulating in the blood.

While the pH of the exudate of vaccinated or tuberculous guinea pigs was almost always definitely, though only slightly, more acid than a similar exudate in normal guinea pigs, and though correspondingly the mononuclears were also more numerous in the exudate of the sensitized animal, the relationship was not an exact one. Thus on the 2nd and 3rd days following the onset of the inflammation the mononuclears had replaced a large number of the granulocytes originally present in the exudate of both normal and sensitized animals. However there was no corresponding reduction in the pH of the exudate, a fact suggesting that other factors also must be responsible for their appearance at the site of inflammation.

Similar observations have been made in rabbits. Table III shows that the mononuclear phagocytes were mobilized at the site of a non-specific inflammation with greater rapidity in a tuberculous than in a normal rabbit. The pH of the exudate, however, did not differ significantly. Obviously, therefore, the pH of the exudate is not the determining factor for the type of cell mobilized. Nor were the cells of the exudate a reflection of those circulating in the blood at that time. Again, as was found in the guinea pig, so also in the rabbit, even on the 3rd day following the onset of the inflammation the mononuclears were still present in greater percentage in the exudate of tuberculous than of normal animals. It is plain, therefore, that both sensitized rabbits and guinea pigs mobilize mononuclear phagocytes

more readily than normal animals, in response not only to specific reinfection but also to a non-related irritant. Furthermore the pH of the exudate bears no constant relationship to the mobilization of these cells.

TABLE III

The pH of the Exudate and Its Leukocytic Formula after an Intrapleural Injection of Aleuronat-Starch, together with the Simultaneous Leukocytic Formula of the Blood in Normal and Tuberculous Rabbits

Interval after injection	Rabbit No.		pH of exudate		Leukocytic formula of exudate				Simultaneous leukocytic formula of blood			
					Normal		Tuberculous		Normal		Tuberculous	
	Normal	Tuberculous	Normal	Tuberculous	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes	Granulocytes	Non-granulocytes
1 day	E2-19	Bii3-12	7.15	7.15	98.5	1.5	83.5	16.5	73.0	27.0	84.0	16.0
" "	E2-18	A3i-3	7.05	7.15	90.5	9.5	60.0	40.0	68.0	32.0	51.0	49.0
" "	G3-18	Di3-13	7.25	7.10	86.5	13.5	74.5	25.5	57.0	43.0	48.0	52.0
" "	17-0	396	7.20	7.25	85.0	15.0	65.0	35.0	22.0	78.0	29.0	71.0
" "	16-9	7	7.20	7.00	84.0	16.0	91.3	8.7	27.0	73.0	48.0	52.0
" "	C-38	A3i-6	6.95	7.20	92.5	7.5	89.0	11.0	86.0	14.0	56.0	44.0
" "	—	A3i-5	—	7.15	—	—	45.0	55.0	—	—	44.0	56.0
" "	52	Bis-2	7.40	7.25	95.0	5.0	59.0	41.0	—	—	—	—
" "	G2-12	4	6.95	7.05	93.6	6.4	71.1	28.9				
" "	G2-19	5	6.95	7.10	90.0	10.0	79.7	21.3				
" "	G2-24	BH3	6.95	7.05	90.6	9.4	54.1	45.9				
" "	G2-28	—	6.95	—	87.2	12.8	—	—				
Average ...			7.09	7.13	90.3	9.7	70.2	29.8	55.5	44.5	51.4	43.6
2 days	G2-12	4	7.10	7.15	60.9	39.1	38.6	61.4				
" "	G2-28	5	6.95	7.05	68.9	31.1	59.4	40.6				
" "	—	BH3	—	7.25	—	—	31.9	63.1				
3 "	G2-19	5	7.35	7.00	23.6	76.4	22.5	77.5				
" "	G2-28	4	7.20	7.15	22.1	77.9	19.8	80.2				
" "	G2-24	BH3	7.25	7.30	36.5	63.5	10.2	89.8				

Capacity for Division and Phagocytosis of Mononuclears Derived from Normal and Immunized Animals

Preliminary observations both in rabbits and guinea pigs indicated that not only do the mononuclears appear earlier at the site of inflammation in a tuberculous than in a normal animal, but the lymphocytes also accumulate more rapidly in the former. It would seem that the entire succession of cells that characterizes inflammation in general

is accelerated in the tuberculous animal irrespective of the nature of the irritant. Again the mononuclears in the exudate of a tuberculous animal more frequently contained ingested polymorphonuclears, which were more often in advanced stages of disintegration than those of a normal animal. This increased phagocytosis, however, may be due not to the enhanced physiological activity of these cells, but to the more rapid deterioration that the polymorphonuclears undergo in the commonly more acid medium of the exudate in the tuberculous animal, as suggested by the work of Evans (15). Hence more of them are available for phagocytosis in the sensitized than in the normal animal, for injured cells are more readily phagocyted than normal ones. Yet, the mobilized mononuclears in the immune animal were often larger and more frequently binucleate than those in the exudates of normal animals. These and other considerations suggested that the differences observed may all be an expression of a heightened physiological activity on the part of these cells in a sensitized animal as compared with that of the mononuclears in a normal animal.

In Vivo Cell Division and Phagocytosis by Mononuclears in Normal and Immunized Animals

To test the foregoing assumption groups of normal, actively tuberculous and R 1 vaccinated guinea pigs were given a mixture of aleuronat-starch and India ink intrapleurally. The injection was made in pairs, a normal and a sensitized animal receiving an identical amount of the same materials. On the 2nd day after the injection the exudates of the sensitized and the normal animals were withdrawn, stained in the usual manner and the following observations made: the percentage cell distribution, the per cent of mononuclears containing ingested carbon, the amount of carbon present per cell, the incidence of binucleated or multinucleated cells and the occurrence of mitotic figures.

Table IV indicates that both tuberculous and vaccinated guinea pigs responded with a greater percentage of mononuclears on irritation with a mixture of aleuronat-starch and India ink than normal animals, just as they reacted to aleuronat alone. A larger percentage of the mobilized mononuclears phagocyted carbon particles than those in a normal guinea pig. The individual mononuclears of the sensitized animals engulfed more carbon than those in the normal animal.

TABLE IV

The Leukocytic Formula, the Intensity of Phagocytosis and the Incidence of Mono-nuclear Division in the Pleural Exudates of Normal and Sensitized Guinea Pigs 2 Days after Injection of Aleuronat-Starch and India Ink

Guinea pig and experiment No.	Tuberculous, vaccinated or normal	Granulo-cytes	Non-granulo-cytes	Mono-nuclears phago-cyting carbon	Phago-cytes with coarse particles or aggluti-nated masses of carbon	Binucle-ated or multi-nucleated mono-nuclears	Presence of mitotic figures
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	
93,1	Tuberculous	47.0	53.0	91.2	79.0	9.2	+
72,1	Normal	97.0	3.0	45.5	46.0	6.0	+
30,2	Tuberculous	57.6	42.4	92.0	87.0	8.0	+
73,2	Normal	94.6	5.4	46.0	45.0	3.0	0
69,3	Tuberculous	64.0	36.0	55.0	70.0	11.5	+
74,3	Normal	93.6	6.4	48.7	49.0	8.2	+
30-4,4	Tuberculous	72.5	27.5	88.0	79.0	28.0	+
38-9,4	Normal	84.5	15.5	28.0	35.7	15.0	0
38-5,4	Normal	82.5	17.5	52.0	46.1	7.0	−
35-7,5	Tuberculous	81.0	19.0	66.0	4.9*	19.0	+
39-7,5	Normal	81.0	19.0	45.0	5.0*	11.0	0
33-6,6	Tuberculous	61.5	38.5	63.0	9.3*	29.0	−
39-6,6	Normal	77.0	23.0	46.0	6.4*	30.0	−
34-9,7	Tuberculous	68.0	32.0	92.0	12.6*	33.0	+
39-8,7	Normal	78.5	21.5	72.0	7.5*	28.0	0
33-5,8	Tuberculous	79.0	21.0	53.0	6.2*	29.0	0
39-5,8	Normal	93.0	7.0	43.0	7.7*	25.0	+
18-3,9	Vaccinated	67.7	32.3	85.6	84.0	7.8	+
70-0,9	Normal?†	67.8	32.2	81.5	71.0	7.2	+
18-5,10	Vaccinated	57.5	42.5	83.5	46.0	16.0	+
30-3,10	Normal‡	61.0	39.0	66.0	69.1	9.0	0
18-7,11	Vaccinated	67.0	33.0	80.0	69.5	21.0	+
30-2,11	Normal‡	72.0	28.0	68.0	63.3	10.0	0
Average	Tuberculous	65.7	34.3	77.2	73.5	19.2	+
	Normal	81.9	18.1	53.5	53.7	13.3	−

* Number of carbon particles per cell.
† Mononuclear nodules with numerous mitoses in the lung; fibrosis of cervical nodes.
‡ Received aleuronat-starch into opposite pleural cavity 3 days before present injection.

This ingested carbon was also found in coarser aggregates in the former (Figs. 1 and 2).   The incidence of both amitotic and mitotic division was greater in the sensitized than in the normal guinea pigs (Fig. 3).

A statistical analysis of the data presented in this table showed that the probability, P, that the observed difference in the phagocytosis of carbon particles was not due to random sampling is 99.9 per cent. This and similar figures reported in this paper were calculated from the "Student" t–distribution curve as given by Fisher (16) and from the tabulated values of P as published by Yule and Kendall (17).

TABLE V

The Leukocytic Formula, the Intensity of Phagocytosis and the Incidence of Mono-nuclear Cell Division in Normal and Tuberculous Rabbits 2 Days after Intrapleural Injection of India Ink and Aleuronat-Starch

Rabbit No.	Normal or tuberculous	Granulocytes	Non-granulocytes	Mononuclears phagocyting carbon	Binucleated or multinucleated mononuclears
		per cent	*per cent*	*per cent*	*per cent*
DM-3	Tuberculous	45.3	54.7	25.0	23.0
20-5	Normal	75.2	24.8	12.5	1.0
Y-4	Tuberculous	43.7	56.3	33.0	8.0
20-7	Normal	63.2	36.8	40.0	1.0
DM-1	Tuberculous	53.7	46.3	33.0	35.0
20-4	Normal	70.3	29.7	16.5	1.5
FM-3	Tuberculous	51.2	48.8	40.7	24.0
20-6	Normal	44.4	55.6	46.5	4.5
Average	Tuberculous	48.5	51.5	34.0	22.5
	Normal	63.3	36.7	28.9	2.0

The value of P from the incidence of binucleated or multinucleated cells is 92.

Similar experiments in normal and tuberculous rabbits are summarized in Table V.   It is seen that in rabbits also the incidence of binucleated or multinucleated mononuclears in the exudate of tuberculous animals was much greater than that found in the exudates of the normal.   The value of P is 99.4.   No significant difference in the phagocytosis of carbon by these cells was found.

Therefore it seems evident that the mononuclears of sensitized guinea pigs and rabbits respond *in vivo* with a greater degree of cell division to a non-specific irritant than those of normal animals.

This increased tendency for division on the part of mononuclears of a tuberculous animal may be held, at least in part, responsible for the more rapid mobilization of these cells in response both to a specific and to a non-specific irritant. That the phagocytic properties of the mononuclears of the sensitized guinea pigs are also increased is suggested by the fact that a greater percentage of these cells are engaged in phagocytosis of carbon *in vivo* and that a majority of these cells in the sensitized animal engulf a larger amount of carbon than the cells in normal animals. However, the evidence is not conclusive, for it has been demonstrated that carbon introduced into the tissues of tuberculous animals agglutinates to a greater degree than in a normal animal (1, 1936). Therefore the enhanced phagocytosis observed may be due to the different dispersion of the carbon particles in the tuberculous or vaccinated animal rather than to the increased physiological capacity for phagocytosis. Hence *in vitro* experiments with cells washed free of their body fluids, and in the presence of serum derived also from normal animals, are essential to test this conclusion.

In Vitro Phagocytosis of Carbon Particles by Mononuclears Derived from Normal and Immunized Guinea Pigs

Accordingly *in vitro* phagocytosis experiments were set up with mononuclears derived from normal, actively tuberculous and vaccinated guinea pigs. The method used is essentially that described by Lucké, Strumia, Mudd, McCutcheon and Mudd (18), with slight modifications.

Briefly, 20 to 40 cc. of light mineral oil were injected intraperitoneally into a normal, an R 1 vaccinated or a tuberculous guinea pig. The injection was executed in pairs, a normal and a sensitized animal receiving an identical amount of the same substance. From 4 to 8 days thereafter 100 cc. sterile 0.4 per cent sodium citrate in 0.85 per cent sodium chloride (19) was injected intraperitoneally. The animal was then quickly killed. The cells were removed and, after separation of the oil, were centrifuged at low speed, decanted, washed in saline and centrifuged again at the same speed. The cells derived from both animals were counted and adjusted with saline to contain the same number per cubic millimeter. Supravital preparations with neutral red were made of each exudate used for the phagocytosis experiments.

Into each of two sterile tubes, 7.5 × 1.0 cm., was pipetted a given volume of cells derived from the normal animal, and into two other tubes was placed the same volume of cells from the immunized animal. To one of each of these pairs

was added an amount of fresh or inactivated serum of a given dilution derived from the normal animal; to the other was added an identical amount of a similar serum of the same dilution derived from the sensitized animal of the same experiment. To each of these four tubes was now added the same volume of the same dilution of India ink. The tubes were stoppered with sterile paraffined corks, and rotated at 37°C. in the incubator on the machine described by Lucké (18) for 20 to 30 minutes at 5 to 8 revolutions per minute. The tubes were then immediately plunged into ice water to stop phagocytosis. Smears were made and stained as previously described. At least 200 cells were counted to determine the percentage distribution of cells in the mixture. A minimum of 100 mononuclears was counted to determine the percentage of cells engaged in phagocytosis of carbon particles, and at least 50 of the cells with ingested particles were examined for the amount of carbon they contained. Table VI presents the essential observations.

It should be noted first in appraising the results that usually the exudates derived from normal animals contained more cells than those derived from the actively tuberculous or vaccinated guinea pigs. The mononuclears of the immunized animals were usually larger, with more abundant cytoplasm, and, in supravital preparations, frequently contained more prominent neutral red granules than those derived from normal animals. Often these vacuoles were tinted more yellow in the "immune" cells, a fact indicating a higher hydrogen ion concentration within their cytoplasm. In many cases the pseudopodia of the immune cells were sensibly more prominent than those of mononuclears derived from normal animals. These observations suggest a greater physiological activity on the part of the mononuclears derived from sensitized animals.

It will be noted in the third column that the number of mononuclears in each phagocytic mixture of a given experiment was similar for the normal and immune cells in the majority of instances. The lymphocytes were usually more numerous in the exudates derived from immunized than those from normal animals, just as was found previously with aleuronat-starch as the inflammatory irritant. This fact tended to lower somewhat the reported percentage of cells engaged in phagocytosis in the mixtures derived from immunized animals. For it was not always possible to differentiate with certainty large lymphocytes, which are rarely phagocytic, from mononuclear phagocytes in stained smears, which were the basis for the estimate of their phagocytic rates.

From the fifth column it is evident that in 9 out of 12 experiments, in the presence of serum derived from the normal animal of a given pair, a significantly larger percentage of mononuclears derived from immunized guinea pigs were engaged in phagocytosis of carbon than mononuclears derived from normal animals under identical conditions, P being 93 and 99 for mononuclears derived respectively from tuberculous and vaccinated guinea pigs. Furthermore, as may be seen from the next column, the amount of carbon ingested by the average immune mononuclear was greater than that engulfed by normal mononuclears.

TABLE VI

In Vitro Phagocytosis of Carbon Particles by Mononuclears Derived from Normal and Sensitized Guinea Pigs

Guinea pig and experiment No.	Tuberculous, vaccinated or normal	Number of mononuclears per c.mm. in phagocytic mixture	Serum dilution	Phagocytosis in normal serum				Phagocytosis in immune serum			
				Mononuclears phagocyting carbon	Number of carbon particles per cell	Number of carbon particles in 100 cells	Ratio between amount of carbon phagocyted by 100 immune and 100 normal cells	Mononuclears phagocyting carbon	Number of carbon particles per cell	Number of carbon particles in 100 cells	Ratio between amount of carbon phagocyted by 100 immune and 100 normal cells
				per cent				*per cent*			
13,1	Tuberculous	12,300	Undiluted	96.0	14.0	1344	1.81	92.0	13.6	1251	0.98
6,1	Normal	5000	"	75.0	9.9	742		85.0	15.1	1283	
84,2	Tuberculous	12,350	"	89.5	22.0	1969	1.99	95.0	26.0	2470	2.29
9,2	Normal	12,800	"	82.5	12.0	990		82.0	13.3	1090	
30-0,3	Tuberculous	19,200	1:10	85.0	13.3	1130	3.91	96.0	16.4	1574	5.14
43-7,3	Normal	18,250	"	85.0	3.4	289		68.0	4.5	306	
36-0,4	Tuberculous	11,500	""*	97.0	17.1	1658	2.07	95.0	17.0	1615	3.04
41-0,4	Normal	14,300	""*	87.0	9.2	800		78.0	6.8	530	
36-2,5	Tuberculous	22,900	""*	97.0	11.0	1067	1.09	90.0	7.3	657	1.77
41-3,5	Normal	30,600	""*	96.0	10.2	979		79.0	4.7	371	
37-0,6	Tuberculous	36,000	""*	86.0	4.3	369	1.57	93.0	3.1	288	0.98
42-0,6	Normal	36,000	""*	90.0	2.6	234		84.0	3.5	291	
36-1,7	Tuberculous	3900	Undiluted*	82.0	18.5	1517	1.74	83.0	18.2	1510	1.61
41-1,7	Normal	8800	""*	67.0	13.0	871		72.0	13.0	936	
18-6,8	Vaccinated	12,200	"	94.5	†			92.0	†		
78,8	Normal	17,700	"	75.0				65.0			
18-2,9	Vaccinated	7500	"	79.0				84.8			
40-1,9	Normal	8700	"	67.6				76.9			
18-1,10	Vaccinated	18,170	"	85.0				92.2			
40-1,10	Normal	8700	"	67.6				76.9			
17-8,11	Vaccinated	9800	"	82.5	†			87.3	†		
79,11	Normal	10,100	"	68.9				70.1			
19-0,12	Vaccinated	20,700	1:10	93.0	6.7	656	1.19	95.0	9.1	864	1.93
43-6,12	Normal	15,800	"	85.0	6.5	552		86.0	5.2	447	

* Serum inactivated at 56°C. for ½ hour.

† On the average these cells contained more and coarser carbon particles than those of their corresponding controls.

The final estimate of the phagocytic capacity of these cells was obtained by determining the number of particles that were ingested by 100 unselected mononuclears derived from the normal and the immunized animal of a given experiment. This resulted from the multiplication of the percentage of cells engaged in phagocytosis by the observed average number of particles ingested, and is recorded in the seventh column. It is plain that this estimate is more significant than either the percentage of cells engaged in phagocytosis, or the average number of ingested particles per cell alone would be. For in any condition in which the fate of an animal depends on the phagocytic activity of its cells, it is not only the number of cells that are capable of phagocytosis, but also the amount of material that each of these cells will engulf, that will determine the outcome.

In the next column, the ratio between the amount of carbon phagocyted by 100 normal and 100 immune mononuclears is given. It is clearly seen that in the majority of instances a markedly greater capacity for phagocytosis is apparent in mononuclears derived from tuberculous or vaccinated animals as compared with that of mononuclears derived from normal animals. This ratio is graphically presented in Text-fig. 1.

Essentially similar observations are recorded in the remainder of Table VI in relation to these same phagocytic mixtures, but in the presence of the serum of the sensitized animal of the given pair. The value of P for the percentage phagocytosis by mononuclears derived from actively tuberculous and vaccinated animals is 99 for both. Figs. 4 and 5 illustrate the *in vitro* phagocytosis of carbon particles by cells derived from a tuberculous and a normal guinea pig in the presence of the serum of the tuberculous individual of the experimental pair. In Text-fig. 1 are also graphically presented the ratios between the amount of carbon phagocyted by 100 unselected normal mononuclears and 100 immune mononuclears in the presence of the immune serum of the tuberculous partner of each test.

In summary, therefore, the phagocytic capacity of mononuclears derived from tuberculous or R 1 vaccinated guinea pigs for non-specific particulate matter such as carbon is, in the majority of instances, significantly greater than that of phagocytes derived from normal animals. Out of a total of 14 experiments with 7 tuberculous guinea pigs and their normal controls in the presence of normal and immune serum, the mononuclears derived from the tuberculous animals were significantly more actively phagocytic than those derived from normal animals in 11, or 78.6 per cent. In the remaining 3 experiments there was no significant difference in the phagocytic capacities of leukocytes derived from the normal and the tuberculous individuals.
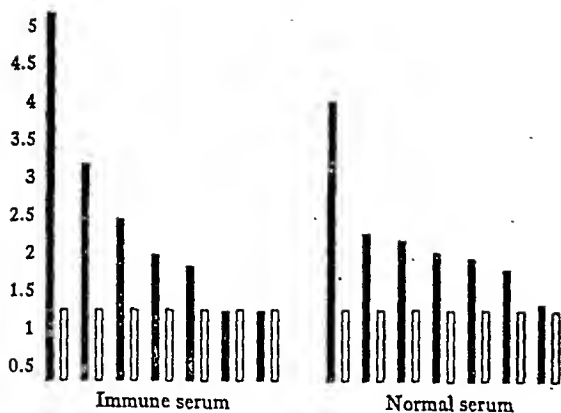
TEXT-FIG. 1

TEXT-FIG. 2

TEXT-FIG. 1. Ratio between the number of carbon particles phagocyted by 100 unselected mononuclears derived from normal and tuberculous guinea pigs in the presence of immune serum and normal serum.

☐ mononuclears derived from normal animals.

▮ mononuclears derived from sensitized animals.
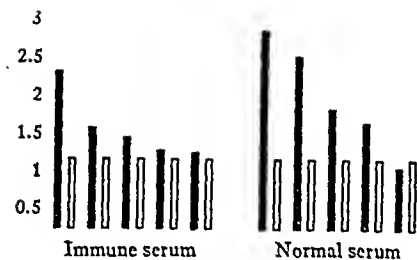
TEXT-FIG. 2. Ratio between the number of staphylococci phagocyted by 100 unselected mononuclears derived from normal and tuberculous rabbits in the presence of immune serum and normal serum.
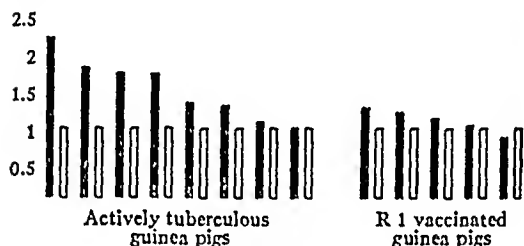
TEXT-FIG. 3

TEXT-FIG. 4

TEXT-FIG. 3. Ratio between the number of tubercle bacilli phagocyted, in the presence of immune serum, by 100 unselected mononuclears derived from normal guinea pigs and from actively tuberculous guinea pigs and R 1 vaccinated guinea pigs.
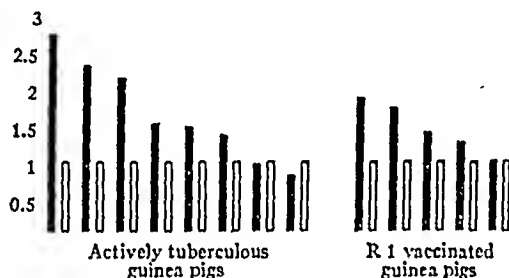
TEXT-FIG. 4. Ratio between the number of tubercle bacilli phagocyted, in the presence of normal serum, by 100 unselected mononuclears derived from normal guinea pigs and from actively tuberculous guinea pigs and R 1 vaccinated guinea pigs.
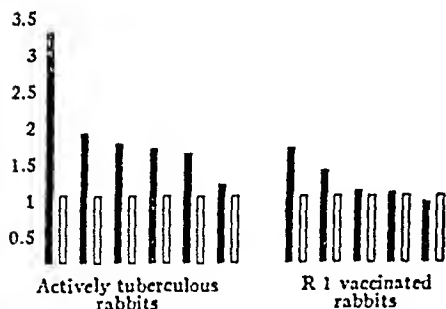
TEXT-FIG. 5

TEXT-FIG. 6

TEXT-FIG. 5. Ratio between the number of tubercle bacilli phagocyted, in the presence of immune serum, by 100 unselected mononuclears derived from normal rabbits and from actively tuberculous rabbits and R 1 vaccinated rabbits.
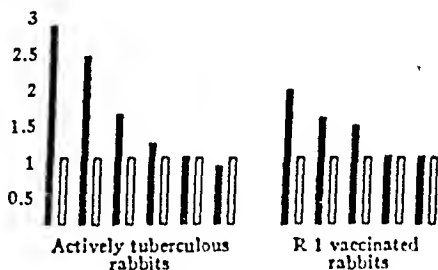
TEXT-FIG. 6. Ratio between the number of tubercle bacilli phagocyted, in the presence of normal serum, by 100 unselected mononuclears derived from normal rabbits and from actively tuberculous rabbits and R 1 vaccinated rabbits.

In 10 additional experiments with guinea pigs vaccinated with R 1 and controls there was a uniformly greater percentage of mononuclears engaged in phagocytosis when they were derived from vaccinated than when obtained from normal animals. Incomplete observations in this series indicated that the individual mononuclears of vaccinated animals also tended to engulf more carbon particles than individual mononuclears derived from normal animals.

In Vitro Phagocytosis of Staphylococci and Collodion Particles by Mononuclears Derived from Normal and Immunized Rabbits

In vitro phagocytosis trials with rabbit mononuclears and India ink gave no satisfactory results. Therefore experiments were set up with collodion particles and staphylococci as phagocytic material. These were carried out in exactly the same manner as was described above for phagocytosis of carbon particles by guinea pig cells except that the rabbits received larger amounts of mineral oil to elicit the mononuclear exudate and more citrated saline was used to wash the peritoneal cavity. Smears of phagocytic mixtures with staphylococci were prepared with Wright's stain, as described above, while those containing collodion particles were stained with the Ziehl-Neelsen technique, as the latter are acid-fast.

Table VII presents the pertinent observations on the relative capacity for phagocytosis of unrelated particles, staphylococci, by mononuclears derived from normal rabbits and from rabbits infected with virulent bovine tubercle bacilli, in the presence of the sera derived from these normal and tuberculous rabbits. Similar observations with collodion as the non-specific test particles for phagocytosis are also presented in Table VII.

It is clear that mononuclears derived from tuberculous rabbits usually have a greater phagocytic capacity for staphylococci in the presence of either normal or immune serum than these cells possess when obtained from normal animals. The value of P for the number of staphylococci phagocyted by 100 unselected mononuclears is 92 and 85 in the presence of normal and immune serum respectively. Likewise immune mononuclears coming from rabbits vaccinated with R 1 ingest a greater amount of collodion particles than these phagocytes do when derived from normal rabbits. The latter conclusion is less certain statistically, for the values of P here are only 61 and 87 for the same observations. Yet, in 3 out of 4 rabbits the mononuclears derived from the vaccinated animals ingested from 2 to 6 times the number of collodion particles phagocyted by cells derived from normal animals. Text-fig. 2 graphically presents the results with

staphylococci.   Figs. 6 and 7 illustrate the relative phagocytosis of staphylococci by mononuclears derived from tuberculous and normal

TABLE VII

In Vitro Phagocytosis of Staphylococci and Collodion Particles by Mononuclears Derived from Actively Tuberculous, R 1 Vaccinated and Normal Rabbits

Rabbit and experiment No.	Tuberculous, vaccinated or normal	Number of mononuclears per c.mm. in phagocytic mixture	Serum dilution	Phagocytosis in normal serum				Phagocytosis in immune serum			
				Mononuclears phagocyting particles	Number of particles per cell	Number of particles in 100 cells	Ratio between number of particles phagocyted by 100 immune and 100 normal cells	Mononuclears phagocyting particles	Number of particles per cell	Number of particles in 100 cells	Ratio between number of particles phagocyted by 100 immune and 100 normal cells
Staphylococci											
				per cent				*per cent*			
20-7, 1	Tuberculous	11,500	Undiluted	97.8	24.4	2386	2.46	96.0	20.9	2006	2.24
10-6, 1	Normal	9000	"	79.0	12.3	971		81.5	11.0	896	
20-5, 2	Tuberculous	4300	"	99.5	16.1	1601	1.54	97.5	12.1	1179	1.11
10-8, 2	Normal	6600	"	91.0	11.4	1037		91.5	11.6	1061	
15, 3	Tuberculous	2350	"	88.0	12.7	1117	2.83	83.0	11.5	954	1.42
5, 3	Normal	3400	"	73.0	5.4	394		79.0	8.5	671	
AD, 4	Tuberculous	3900	"	80.0	10.8	864	1.72	79.0	9.6	758	1.30
21-3, 4	Normal	3000	"	69.0	7.3	503		60.0	9.7	582	
20-8, 5	Tuberculous	12,500	"	59.0	5.6	330	0.89	58.0	6.6	382	1.09
21-2, 5	Normal	14,400	"	63.0	5.9	371		55.0	6.4	352	
Collodion Particles											
33-3, 1	Vaccinated	5700	Undiluted	79.0	2.9	229	4.98	74.0	3.0	222	2.13
33-2, 1	Normal	5600	"	33.0	1.4	46		55.0	1.9	104	
33-4, 2	Vaccinated	49,280	1:10	32.0	3.7	118	4.21	61.0	3.1	189	2.45
33-5, 2	Normal	52,080	"	19.0	1.5	28		43.0	1.8	77	
33-7, 3	Vaccinated	40,000	"	92.5	6.6	610	4.04	97.0	11.3	1096	6.68
33-6, 3	Normal	25,000	"	63.0	2.4	151		71.5	2.3	164	
34-1, 4	Vaccinated	49,820	"	93 5	7.2	673	0 64	70 0	5 9	413	1 05
34-2, 4	Normal	46,375	"	91.0	11 5	1046		87 5	4 5	393	

rabbits in the presence of the latter's serum.   Figs. 8 and 9 illustrate the phagocytosis of collodion particles by the mononuclears of a vaccinated and a normal rabbit in the presence of the former's serum.

In Vitro Phagocytosis of Tubercle Bacilli by Mononuclears Derived from Normal and Immunized Guinea Pigs and Rabbits

It is evident, therefore, that the phagocytic capacity of mononuclears derived from actively tuberculous or vaccinated rabbits and guinea pigs for substances unrelated to the disease is greater than that of phagocytes derived from normal animals, and that this is independent of the presence of normal or immune serum; in both media the phagocytes of the immunized animals ingest more particles. From the standpoint of the mechanism of immunity to tuberculosis it is important to determine whether the enhanced phagocytic capacity conferred by active tuberculosis or by vaccination upon these mononuclears applies also to the tubercle bacillus. Accordingly similar experiments were set up with mononuclears and sera derived from normal, actively tuberculous and R 1 vaccinated guinea pigs and rabbits, using living virulent human type tubercle bacilli (P 15 B) as the particle for phagocytosis.

The experiments were set up in the manner described above in detail for carbon particles. The serum, fresh or inactivated, in different dilutions, constituted one-third of the volume of the phagocytic mixtures. There were between 150,000 and 580,000 tubercle bacilli per c.mm. in the phagocytic mixtures of the different experiments. It is needless to state that in any test of the properties of normal and immune cells all the conditions were as nearly alike as was possible; the single known variable was the derivation of the phagocytes.

Table VIII presents the pertinent data for cells derived from guinea pigs. In columns 3 to 5 are included the differential cell counts of all the exudates that resulted in each one of the normal and sensitized guinea pigs from the injection of the same amount of petrolatum after the same lapse of time. It will be noted that, in general, sensitized animals, whether actively tuberculous or vaccinated, responded to the intraperitoneal injection of mineral oil with an exudate that contained a larger percentage of mononuclears and lymphocytes and a conspicuously lower concentration of granulocytes than did normal animals. In other words, as was noted above with aleuronat-starch as an irritant, the succession of cells that characterizes inflammation in general was accelerated in the immunized animal in reaction to this non-specific irritant also.

It is clear from an analysis of the remaining observations recorded

TABLE VIII

In Vitro Phagocytosis of Tubercle Bacilli by Mononuclears Derived from Normal, Actively Tuberculous and R 1 Vaccinated Guinea Pigs

Experiment No.	Tuberculous, vaccinated or normal	Differential count of cells in exudate			Number of mononuclears per c.mm. in phagocytic mixture	Serum dilution	Phagocytosis in normal serum			Phagocytosis in immune serum		
		Mononuclears	Lymphocytes	Granulocytes			Mononuclears phagocyting tubercle bacilli	Number of tubercle bacilli per cell	Ratio between number of tubercle bacilli phagocyted by 100 immune and 100 normal cells	Mononuclears phagocyting tubercle bacilli	Number of tubercle bacilli per cell	Ratio between number of tubercle bacilli phagocyted by 100 immune and 100 normal cells
		per cent	per cent	per cent			per cent			per cent		
1	Tuberculous	63.0	36.0	1.0	12,350	Undiluted	86.0	4.8	1.38	81.0	5.3	1.32
	Normal	65.5	13.5	21.0	12,800	"	68.0	4.4		72.0	4.5	
2	Tuberculous	82.5	12.0	5.5	12,300	"	85.0	13.7	1.55	79.0	10.1	1.00
	Normal	33.5	1.5	65.0	5000	"	84.0	8.9		82.0	9.8	
3	Tuberculous	74.0	8.0	18.0	18,000	"	79.0	9.2	1.50	63.0	9.3	1.36
	Normal	74.0	3.0	23.0	17,000	"	69.0	7.0		58.0	7.4	
4	Tuberculous	93.5	5.5	1.0	19,200	1:10	66.0	7.2	2.20	69.0	11.3	1.76
	Normal	89.5	5.0	5.5	18,250	"	47.0	4.6		59.0	7.5	
5	Tuberculous	65.4	31.2	3.4	11,500	"•	34.0	2.0	2.83	91.0	7.9	1.85
	Normal	78.0	2.5	19.5	14,300	"•	12.0	2.0		76.0	5.1	
6	Tuberculous	66.3	22.0	11.7	22,900	"•	38.0	2.8	0.96	66.0	4.5	1.09
	Normal	72.7	4.6	22.7	30,600	"•	37.0	3.0		62.0	4.4	
7	Tuberculous	74.5	18.0	7.5	36,000	"•	28.0	1.9	0.80	31.0	4.1	2.27
	Normal	61.1	8.4	30.5	36,000	"•	33.0	2.0		28.0	2.0	
8	Tuberculous	73.0	11.0	16.0	3900	Undiluted*	93.0	26.5†	2.39	91.0	23.4	1.77
	Normal	80.0	12.5	7.5	8800	"•	66.0	15.6		80.0	15.0	
9	Vaccinated‡	83.5	6.5	10.0	29,100	1:50	72.0	3.3	1.93	51.0	3.6	1.03
	Normal	35.5	2.0	62.5	11,500	"	41.0	3.0		47.0	3.8	
10	Vaccinated	68.5	22.5	9.0	30,140	1:10	61.0	4.6	1.42	71.0	6.3	1.23
	Normal	67.5	15.5	17.0	29,700	"	45.0	4.4		65.0	5.6	
11	Vaccinated	82.0	6.0	12.0	25,500	"	83.0	7.7	1.29	76.0	11.7	1.30
	Normal	73.0	4.5	22.5	22,700	"	73.0	6.8		76.0	9.0	
12	Vaccinated	60.5	17.5	22.0	26,100	"	64.0	5.6	1.79	90.0	11.4	0.87
	Normal	61.3	12.0	26.7	27,300	"	50.0	4.0		85.0	13.9	
13	Vaccinated	82.0	11.5	6.5	20,700	"	33.0	2.2	1.01	71.0	7.7	1.14
	Normal	61.5	7.0	31.5	15,800	"	29.0	2.5		60.0	8.0	
Average	Sensitized	74.5	16.0	9.5								
	Normal	65.6	7.1	27.3								

* Serum inactivated at 56°C. for ½ hour.

† Estimated, not directly counted because of agglutination of ingested bacilli.

‡ Vaccinated with suspension of tubercle bacilli killed by submersion in liquid petrolatum at 37°C. for 8 days. The guinea pig's sensitivity to tuberculin was + +.

in this table that the phagocytic capacity for tubercle bacilli of mononuclears derived respectively from actively tuberculous and vaccinated guinea pigs was, in a large majority, greater in the presence of either normal or immune serum than that of mononuclears obtained from normal animals, under identical conditions. In the remaining instances there was no distinct difference in the phagocytosis of tubercle bacilli by mononuclears derived from either normal or immunized guinea pigs.

A further analysis of these data shows that the observed enhancement of the phagocytic capacity of mononuclears conferred upon them by active tuberculosis has a probability of significance of 83 per cent for the number of tubercle bacilli phagocyted by 100 unselected mononuclears in the presence of either normal or immune serum.[1] However, the observed smaller increment in phagocytic capacity afforded mononuclears by vaccination is of questionable statistical significance. The value P for the corresponding observations is only 77 and 57 in the presence of normal and immune serum respectively with cells derived from such animals.

These results are graphically presented in Text-figs. 3 and 4. Figs. 10 and 11 illustrate the relative phagocytosis of tubercle bacilli by mononuclears derived from tuberculous and normal guinea pigs in the presence of the former's serum.

In Table IX are presented the pertinent data for the relative phagocytosis of tubercle bacilli by mononuclears derived from normal, actively tuberculous and R 1 vaccinated rabbits. It is evident from an examination of these protocols, that the phagocytic capacity for tubercle bacilli of mononuclears derived from tuberculous or vaccinated rabbits is greater in the majority of instances than that of mononuclears derived from normal rabbits in the presence of either normal or immune serum. In the remaining instances there was no significant difference in phagocytosis between these mononuclears. It is also apparent that active tuberculosis frequently confers upon the mononuclears a relatively greater enhancement of their phagocytic

[1] For the sake of brevity this column was omitted from Table VIII; however, the observations from which the data in this column were calculated are given there.

TABLE IX

In Vitro Phagocytosis of Tubercle Bacilli by Mononuclears Derived from Normal, Actively Tuberculous and R 1 Vaccinated Rabbits

Rabbit and experiment No.	Tuberculous, vaccinated or normal	Number of mononuclears per c.mm. in phagocytic mixture	Serum dilution	Phagocytosis in normal serum				Phagocytosis in immune serum			
				Mononuclears phagocyting tubercle bacilli	Number of tubercle bacilli per cell	Number of tubercle bacilli in 100 cells	Ratio between number of tubercle bacilli phagocyted by 100 immune and 100 normal cells	Mononuclears phagocyting tubercle bacilli	Number of tubercle bacilli per cell	Number of tubercle bacilli in 100 cells	Ratio between number of tubercle bacilli phagocyted by 100 immune and 100 normal cells
				per cent				*per cent*			
14, 1	Tuberculous	12,000	Undiluted	84.0	5.1	428	1.65	82.0	5.0	410	1.77
8	Normal	18,000	"	65.0	4.0	260		70.0	3.3	231	
15, 2	Tuberculous	2000	"	85.0	7.7	654	1.22	74.0	12.1	895	1.64
5	Normal	3000	"	80.0	6.7	536		79.0	6.9	545	
FM4, 3	Tuberculous	29,000	"	61.0	2.7	164	1.01	72.0	3.2	230	1.17
7	Normal	27,000	"	49.0	3.3	161		48.0	4.1	196	
16, 4	Tuberculous	10,000	1:10	98.0	13.2	1293	2.96	93.0	11.7	1088	1.70
31-8	Normal	14,000	"	78.0	5.6	436		76.0	8.4	638	
10-4, 5	Tuberculous	9500	Undiluted*	56.0	9.0	504	0.87	83.0	14.0	1162	1.92
10-8	Normal	9000	"*	72.0	8.0	576		65.0	9.3	604	
10-5, 6	Tuberculous	18,500	"*	58.0	8.2	475	2.51	72.0	10.3	741	3.40
10-9	Normal	18,500	"*	43.0	4.4	189		42.0	5.2	218	
33-3, 7	Vaccinated	5700	1:10	81.0	8.4	680	2.03	95.0	10.0	950	1.71
33-2	Normal	5600	"	67.0	5.0	335		73.0	7.6	554	
33-9, 8	Vaccinated	39,000	"	53.0	3.0	159	1.60	63.0	4.1	258	1.07
33-8	Normal	41,000	"	43.0	2.3	99		65.0	3.7	240	
33-4, 9	Vaccinated	49,280	"	68.0	7.9	537	1.49	78.0	5.6	437	1.37
33-5	Normal	52,080	"	60.0	6.0	360		69.0	4.6	317	
33-7, 10	Vaccinated	40,000	"	81.0	14.6	1182	1.02	85.5	17.4	1487	0.90
33-6	Normal	25,135	"	66.5	17.4	1157		72.0	23.0	1656	
34-1, 11	Vaccinated	49,820	"	69.0	8.1	558	1.02	61.9	9.9	612	1.01
34-2	Normal	46,375	"	59.0	9.3	548		69.6	8.8	612	

* Serum inactivated at 56°C. for 30 minutes.

capacities than is afforded them by vaccination with attenuated tubercle bacilli.

When these data are subjected to a statistical analysis it is found that the observed enhancement of the phagocytic capacity conferred upon mononuclears of rabbits by active tuberculosis has a probability of significance of 88 and 96 per cent for the number of tubercle bacilli phagocyted by 100 unselected mononuclears in the presence of normal and immune serum respectively. As was found above with guinea pig cells, however, the observed lesser increment in phagocytic capacity afforded rabbit mononuclears by vaccination of rabbits with attenuated tubercle bacilli is of low statistical significance. The value of P for the corresponding observations is only 68 and 57 in the presence of normal and immune serum respectively.

Text-figs. 5 and 6 summarize the data graphically. Figs. 12 and 13 illustrate the relative phagocytosis of tubercle bacilli by rabbit mononuclears derived from an actively tuberculous and normal animal respectively in the presence of the former's serum.

SUMMARY AND DISCUSSION

An endeavor has been made to elucidate the mechanism of the more rapid mobilization of mononuclear phagocytes that characterizes the response to reinfection of the tuberculous or allergic animal, as distinguished from the reaction to a primary inoculation. It has been found that actively tuberculous or vaccinated rabbits and guinea pigs react with an accelerated appearance of mononuclears in response to non-specific inflammatory irritants also. Furthermore the entire succession of cells that characterizes inflammation in general is accelerated in the allergic animal in reaction to irritants, such as aleuronat-starch or mineral oil; *i.e.* in an allergic animal the replacement of the first mobilized polymorphonuclears by mononuclears and the latter by lymphocytes takes place more rapidly than in a normal animal.

This more rapid mobilization of mononuclears is not accounted for by the more acid reaction which frequently develops at the site of introduction of these irritants in a tuberculous as compared with that in a normal animal. This result was not expected for, as considered above, in view of the pH range for the optimum activity of some of the proteases of the polymorphonuclear and mononuclear cells respec-

tively, one might reasonably anticipate such a relationship. That the higher hydrogen ion concentration often found at the site of inflammation in the allergic animal may be more inimical to the survival of polymorphonuclears than of mononuclears is considered likely, but their mobilization seems to be controlled by other factors. For in rabbits there is no significant difference in the pH level at the site of a non-specific inflammation between the normal and the sensitized animals; yet the mononuclears are present in larger numbers in the latter. Again, even in the guinea pig, while there is a consistently greater acidity at the site of inflammation in the allergic animal, there is no consistent correlation between the leukocytic formula of the exudate and its pH level. Nor does the greater local tissue acidosis that develops at the site of a specific reinfection, as compared with that of a primary inoculation, and as observed with the agar focus technique, account for the inhibition of growth of tubercle in the former. For the pH difference, an average of 0.2, seems insufficient for the effects noted on the tubercle bacillus, the pH growth range of which is so wide. It is significant that a similar slightly greater acidity was found within collodion-impregnated silk bags containing tubercle bacilli placed in the peritoneal cavity of tuberculous, as compared with the contents of similar bags situated in normal rabbits (11). In this instance also the observed inhibition of growth in the body fluids of the immune animal seems unexplained by the slight pH difference. No explanation for this phenomenon has thus far been found.

However, definite data have been obtained which seem to explain the more accelerated inflammatory response that characterizes the immunized animal. In the first place the very fact that non-specific irritants also bring about an acceleration of the succession of cells that characterize inflammation in general in the tuberculous animal suggests that this may be based on a heightened physiological activity on the part of the sensitized animal. Again the mononuclears at the site of a non-specific inflammation in the tuberculous animal are larger, have more abundant cytoplasm, and in supravital smears, frequently contain more numerous and prominent neutral red stained vacuoles, which are often of a yellower tint than those of mononuclears mobilized

in the normal animal. Their pseudopodia also are in many cases more prominent. These differences all suggest an increased physiological activity on the part of the immune phagocytes. Furthermore the rate of both mitotic and amitotic division of mononuclears is conspicuously greater in the exudate of immunized rabbits and guinea pigs than in that of normal animals. It would seem that it is this increased rate of division that is a significant factor in their more rapid mobilization in the immunized animal in response to both specific and non-specific irritants.

Evidence was obtained suggesting an increase in the phagocytic capacity of the immune cells for carbon particles *in vivo*. This, however, was not conclusive. But *in vitro*, unequivocal and statistically significant observations indicate that, in the presence of normal or immune serum, mononuclears derived from tuberculous or vaccinated guinea pigs ingest more carbon particles than mononuclears obtained from normal animals. Mononuclears originating in tuberculous rabbits ingest more staphylococci than do these phagocytes when obtained from normal animals. A similar, though statistically less significant increment in the phagocytic capacity of mononuclears for collodion particles was observed in cells derived from vaccinated rabbits. Thus, the physiological phagocytic activity of the mononuclears of tuberculous rabbits and guinea pigs is enhanced non-specifically in relation to substances that have no bearing on the tuberculous process.

Furthermore mononuclears derived from tuberculous rabbits and guinea pigs exhibit an enhancement in their phagocytic capacity for virulent tubercle bacilli in the presence of both normal and immune serum which is of fairly good statistical significance.

It is clear, therefore, that what has been inferred from the studies on the correlation between the fate of the bacilli and the host responses *in vivo*, namely that immunity to tuberculosis rests upon an increased physiological capacity of the mononuclear phagocytes to destroy or inhibit the growth of tubercle bacilli, has found a suggestive confirmation in these *in vitro* studies. For it is plain that tuberculosis confers upon the mononuclears an increased capacity to divide in response

to irritation, and a capacity to ingest a greater amount of particles available for phagocytosis, whether these be non-specific substances, such as carbon particles, staphylococci or collodion, or specific tubercle bacilli.

It is significant in this relation that the enhancement of the phagocytic capacity for tubercle bacilli afforded mononuclears by a virulent active tuberculosis is greater than that conferred upon them by vaccination with avirulent organisms. Indeed the increment noted in the latter is hardly of any statistical significance. This parallels the well known fact that active tuberculosis confers a greater relative immunity to reinfection than vaccination with an organism of low virulence.

The limited observations of a more acid pH within the cytoplasm of immune mononuclear phagocytes suggest that, just as their propensity to divide and to phagocyte is increased by the presence of tuberculosis, so may it be with their digestive capacities. This would lead to the more rapid destruction or more effective inhibition of the growth of tubercle bacilli within their cytoplasm, as has been previously concluded.

It is interesting in this connection that Metchnikoff, the founder of the theory of phagocytosis, originally held that the increased phagocytosis observed on reinoculation of an animal that had recovered from an acute infectious disease was due to an enhancement of the ingesting capacity of the phagocytes (20). It was soon shown, however, that in these conditions, the increased phagocytosis was the result of specific bacteriotropins present in the serum of the immunized animal and could be elicited with phagocytes obtained from a normal animal. It is clear from this study that in tuberculosis, beside the phagocytosis-promoting antibodies that accumulate in the blood, there also develops a generalized increased physiological activity on the part of these cells, one of which is an increased phagocytic capacity for specific as well as for non-specific substances.

These observations throw light on a number of obscure phenomena. Lewis and Loomis (21) have observed that tuberculous guinea pigs generate antibodies more rapidly than normal animals similarly

stimulated. Dienes (22) discovered that the presence of tuberculous lesions greatly intensifies the pre-anaphylactic sensitizing capacity of antigens. It has been claimed by some observers (23) that tuberculous animals resist other infections with greater efficiency than nontuberculous individuals.

It is possible that these observations are expressions of the enhanced physiological activity on the part of the mesenchymal cells as demonstrated in this study. For in the production of antibodies and in resistance to infections, the rôle of these cells is certainly significant.

CONCLUSIONS

1. Tuberculous and vaccinated rabbits and guinea pigs mobilize mononuclear phagocytes at the site of a non-specific inflammation with greater rapidity than do normal animals, just as they respond to tubercle bacilli.

2. The succession of cells that characterizes inflammation in general is accelerated in allergic rabbits and guinea pigs in response to nonspecific irritants.

3. The pH at the site of reinfection with tubercle bacilli in immunized rabbits and guinea pigs and at the site of a non-specific inflammation in the latter is slightly lower than in a similar site in a normal animal.

4. No constant relation was found between the mobilization of mononuclears and the hydrogen ion concentration at the site of inflammation.

5. The rate of mitotic and amitotic division of mononuclears in allergic rabbits and guinea pigs in response to non-specific irritants is greater than in normal animals.

6. Mononuclears derived from actively tuberculous or vaccinated guinea pigs exhibit greater *in vitro* phagocytic capacity for carbon particles than mononuclears obtained from normal animals.

7. Mononuclears of tuberculous rabbits ingest more staphylococci than the phagocytes of the same type originating from normal animals.

8. Mononuclears originating from actively tuberculous rabbits and guinea pigs exhibit greater *in vitro* phagocytic capacity for tubercle bacilli than mononuclears obtained from normal animals.

9. The enhancement of the phagocytic capacity for tubercle bacilli afforded mononuclears by vaccination with a bacillus of low virulence is lower, and of questionable significance.

10. The increased phagocytic activity of mononuclears derived from tuberculous or vaccinated rabbits and guinea pigs for tubercle bacilli and for non-specific particulate matter occurs in media containing sera derived from normal and from tuberculous individuals.

11. The more rapid mobilization of mononuclears by immunized animals in response to specific as well as non-specific irritants is associated with their increased physiological activity.

The significance of this enhanced activity conferred by the tuberculous process on the mesenchyme cells is discussed in relation to the mechanism of immunity to tuberculosis and other phenomena.

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EXPLANATION OF PLATES

Smears depicted in Figs. 1 to 7 inclusive were prepared with Wright's stain. Those depicted in Figs. 8 to 13 inclusive were stained with the Ziehl-Neelsen procedure.   The magnifications are about × 1400.

PLATE 35

FIG. 1. *In vivo* phagocytosis of carbon particles by mononuclears of tuberculous guinea pig 30.

FIG. 2. *In vivo* phagocytosis of carbon particles by mononuclears of normal guinea pig 73.

FIG. 3. Mitotic and amitotic division of mononuclears in exudate of tuberculous guinea pig 93.

FIG. 4. *In vitro* phagocytosis of carbon particles by mononuclears of tuberculous guinea pig 36-1 in the presence of its own serum.

FIG. 5. *In vitro* phagocytosis of carbon particles by mononuclears of normal guinea pig 41-1 in the presence of the serum of tuberculous guinea pig 36-1, the cells of which are shown in Fig. 4.

FIG. 6. *In vitro* phagocytosis of staphylococci by mononuclears of tuberculous rabbit 20-7 in the presence of serum of normal rabbit 10-6, the cells of which are shown in Fig. 7.

FIG. 7. *In vitro* phagocytosis of staphylococci by mononuclears of normal rabbit 10-6 in the presence of its own serum.

PLATE 35

1

2

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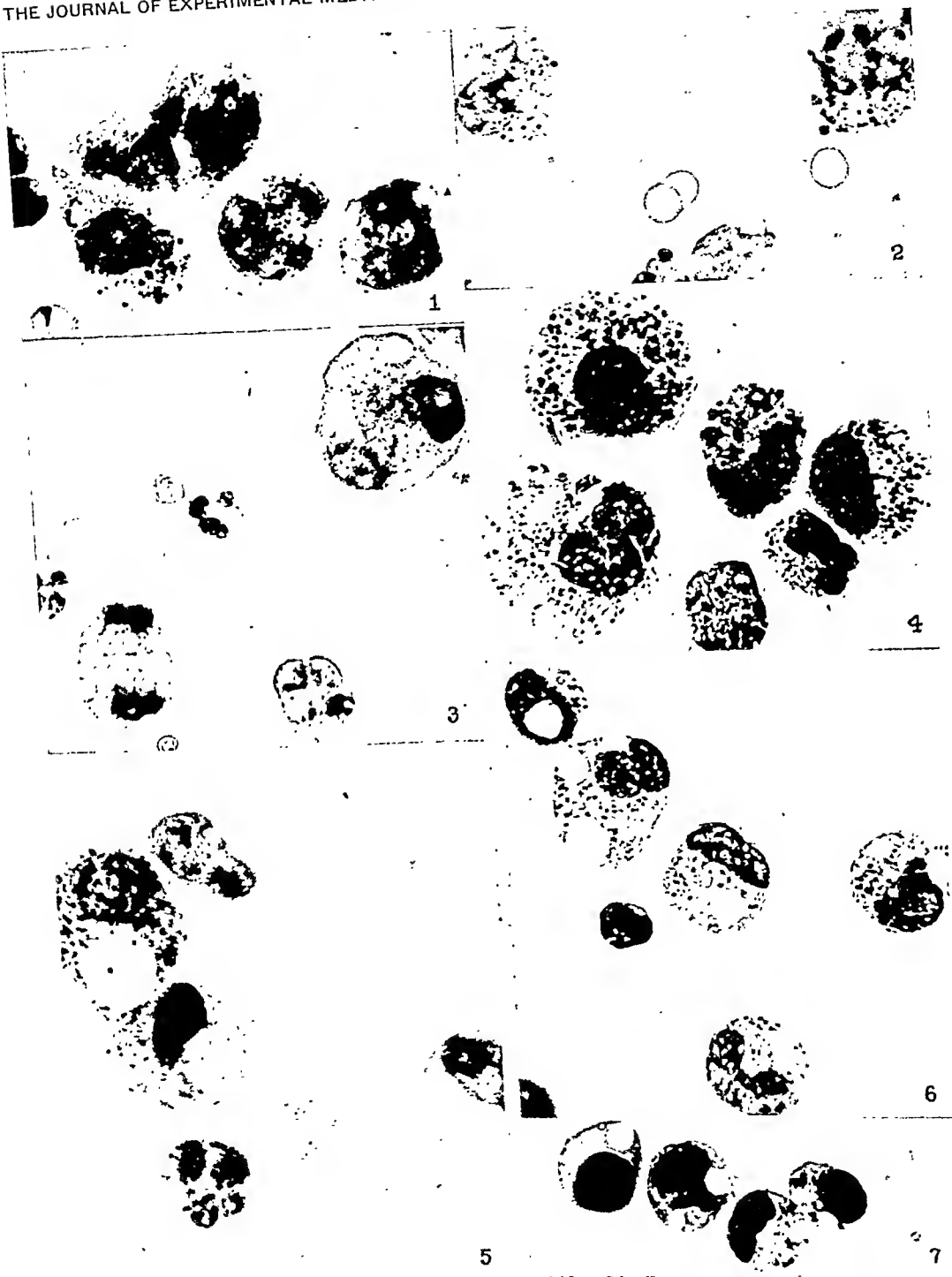
PLATE 36

FIG. 8. *In vitro* phagocytosis of collodion particles by mononuclears of vaccinated rabbit 33-7 in the presence of its own serum.

FIG. 9. *In vitro* phagocytosis of collodion particles by mononuclears of normal rabbit 33-6 in the presence of serum of vaccinated rabbit 33-7, the cells of which are shown in Fig. 8.

FIG. 10. *In vitro* phagocytosis of tubercle bacilli by mononuclears of tuberculous guinea pig 94 in the presence of its own serum.

FIG. 11. *In vitro* phagocytosis of tubercle bacilli by mononuclears of normal guinea pig 30-1 in the presence of serum of tuberculous guinea pig 94, the cells of which are shown in Fig. 10.

FIG. 12. *In vitro* phagocytosis of tubercle bacilli by mononuclears of tuberculous rabbit 16 in the presence of its own serum.

FIG. 13. *In vitro* phagocytosis of tubercle bacilli by mononuclears of normal rabbit 31-8 in the presence of serum of tuberculous rabbit 16, the cells of which are shown in Fig. 12.

PLATE 36
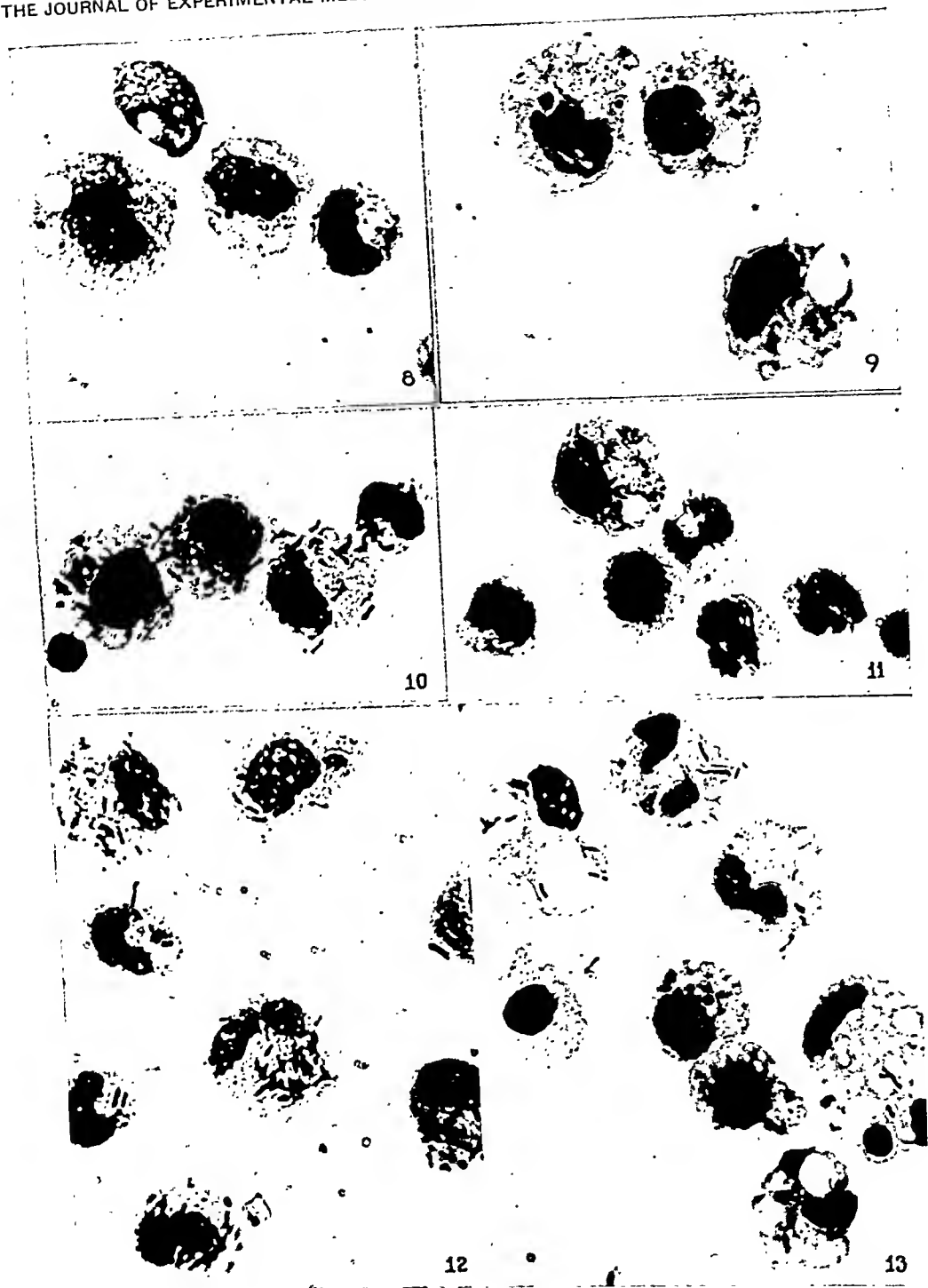
THE MODE OF ACTION OF SULFANILAMIDE ON STREPTOCOCCUS. II*

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It is not surprising, in view of the truly amazing therapeutic results that may follow the use of sulfanilamide and related compounds in streptococcus and other infections, that considerable effort has been devoted to explaining the precise method by which this drug produces its results. The quest is not alone for the purpose of perfecting the drugs employed and their administration, but because in addition such investigations offer a new means of approach to the mechanism of action of chemotherapeutic drugs in general, concerning which we have had far too little information.

A bewildering series of facts are now available that bear on the mode of action of sulfanilamide on microorganisms, both in the test tube and in the animal body. The difficulty in interpreting these facts has been due to over-emphasis on the particular findings of individual investigators on the study of a single factor. Thus there are those who would explain sulfanilamide action as a direct effect upon the microorganism itself, whether acting *in vivo* or *in vitro*. In other words, there are those who would still consider the animal body as a test tube, much as Pasteur explained immunity in the early days of the development of ordered knowledge in this field. There are others, however, who from the beginning have taken cognizance of the importance of the cells of the body in the therapeutic results obtained by sulfanilamide.

The Direct Action of Sulfanilamide on Streptococcus

The effect of sulfanilamide on bacteria cannot be considered from the viewpoint of the action of this substance alone, but in the light of

all other environmental factors that enter into the experiment. In other words, sulfanilamide action depends on the balance of a series of factors that enter into the composition of the medium in which the two interacting substances, sulfanilamide and streptococcus, are tested. We are for the moment primarily considering the substances that enter into the reaction under the artificial conditions as observed in the test tube, leaving for later consideration the additional factors that intervene in the animal body.

Thus we find on analysis from our own experiments and from those of others, that the growth of streptococcus is influenced in the test tube

TABLE I

Factors That Influence the Growth of Streptococci

Favoring	Inhibiting
Good nutrient broth (Infusion broth with best peptone at right pH (7.5))	Poor nutrient broth (Meat extract broth; peptone free broth; pH under 6.5 or over 8)
Glucose	Glucose peptone water (Nitti *et al.*)
Peptone	Washing streptococci (thus removing peptone) (Lockwood, 1938 *b*)
Horse serum	Saline suspension of streptococci (Mellon *et al.*)
Rabbit serum (Gay and Clark)	40°C. (White and Parker)
Defibrinated rabbit blood (Gay and Clark)	Human serum (Colebrook *et al.*)
Defibrinated infant blood (Lyons and Mangiaracine)	Deleucocyted human blood (Colebrook *et al.*, Finklestone-Sayliss *et al.*)
Defibrinated horse blood (White and Parker)	Whole defibrinated human blood (Colebrook *et al.*, Lockwood, 1938 *b*)
Necrotic tissue (peptone ?) (Lockwood, 1938 *a*)	SULFANILAMIDE
	PHAGOCYTES

by two sets of factors: those that favor the growth of the microorganism, and those that inhibit it. It would be impossible to enumerate these growth factors in the precise order of their excellence in favoring or in inhibiting the growth of streptococcus, but they are listed in Table I, more or less in order of their significance.

When tested in any of the favoring media, as listed above, sulfanilamide by general agreement produces bacteriostasis only. Furthermore, the degree of bacteriostasis depends inversely on the sum total of adjuvant growth factors present; thus in a series of comparisons the ratio of growth in the presence of sulfanilamide broth, as compared with plain broth, was as 1–32,500 in 24 hours. The ratio was only

1–10.6 in sulfanilamide rabbit serum, as compared with serum alone, because serum is a better medium.

Experiment 1.—Comparison of Degree of Bacteriostatic Action of Sulfanilamide in Broth and Rabbit Serum.—

Medium	Sulfanilamide 1–5000	Inoculation Streptococcus H	Colonies	
			24 hrs.	48 hrs.
Infusion broth	—	±22	6,500,000	110,000,000
" "	Present	"	200	10
Rabbit serum	—	"	410,000,000	90,000,000
" "	Present	"	38,000,000	6,000,000

On adding certain additional inhibiting factors, none of which alone suffices to destroy the streptococcus, in other words by a summation of injury, sulfanilamide becomes bactericidal. For example, sulfanilamide kills: (1) in poor nutrient broth; (2) by a less nourishing peptone; (3) on removal of peptone by washing streptococci (Lockwood, 1938 *b*); (4) in glucose peptone without meat extract (Nitti *et al.*); (5) after diluting in saline (Mellon *et al.*); (6) at 40°C. (White and Parker); (7) in human serum (Colebrook *et al.*); (8) in whole human blood (Colebrook *et al.*).

To illustrate one of the simplest examples of this summation of injury effect on streptococcus, we may give the growth history in an experiment with our streptococcus H in good and relatively poor peptone media, with and without sulfanilamide.

*Experiment 2.—*An 18 hour infusion broth culture of streptococcus H was inoculated into tubes of two batches of infusion broth of the same pH (7.5), but of which one batch, containing bactopeptone instead of neopeptone, promoted fair but not vigorous growth. To parallel tubes of each kind of broth, sulfanilamide (1–5000) was added. The tubes were plated on 2 successive days and the colonies of streptococcus ascertained:

Infusion broth	Sulfanilamide 1–5000	Inoculation Streptococcus H	Colonies	
			24 hrs.	48 hrs.
Neopeptone	—	±14	350,000,000	80,000
"	Present	"	3800	9000
Bactopeptone	—	"	10,600,000	80,000,000
"	Present	"	70	0

Sulfanilamide thus produced bacteriostasis in the superior (neopeptone) broth and complete destruction in 48 hours in the less adequate (bactopeptone) broth.

If we look on sulfanilamide as only one, although perhaps the most marked of the factors that inhibit test tube growth of streptococci, we may question whether other combinations of unfavorable growth factors might not likewise change bacteriostasis to destruction.   For example, would washed streptococci or saline suspensions of streptococci be killed when grown at 40°C., instead of being merely inhibited at this temperature as they are in the excellent media employed by White and Parker?   We know of no direct answer to such questions.   .

As we pointed out a year ago (Gay and Clark), streptococci inhibited in growth by dilutions of sulfanilamide exhibit involution forms and occur in long chains.   These alterations have been confirmed by Lockwood, 1938 b, and a "clumping" reaction of the lengthened chains added by Meyer, 1938.   Lyons and Mangiaracine confirm all these changes and also the alterations we noted in colonial form.   None of these involution forms are pathognomonic of the action of sulfanilamide and might equally well occur in any unfavorable medium.

Although capsules have long been recognized as characteristic of the virulence of streptococci (Bordet), and in some measure responsible for it, the original explanation of Levaditi and Vaisman, 1935 a, of the mode of action of sulfanilamide as due to interference with capsule formation, has not met with general acceptance (Gay and Clark; Long and Bliss; Colebrook and Kenny).   We continue to find, as already pointed out, capsules on streptococci grown, although inhibited, in the bodies and fluids of sulfanilamide-treated rabbits and mice.   It still remains a possibility, as emphasized by Lyons and Mangiaracine, that there are capsules and capsules, which, although apparently identical morphologically, may differ in antigenic constitution and resistence to phagocytosis.

Far more significant, we believe, is the failure of sulfanilamide to be adsorbed or diminished by the presence of the streptococcus that it affects.   It is true that Finklestone-Sayliss, Paine and Patrick found that a relatively great concentration of fat derived from young streptococci causes sulfanilamide in an aqueous dilution of 1–2000 to diminish

in the watery phase by nearly one-half in 2 hours. This may show that sulfanilamide is relatively more soluble in fat than in water, but under the conditions specified does not, we believe, show that sulfanilamide in its operation on streptococcus is adsorbed by the fat in the microorganism. Bürgers, 1937, states that incubation of streptococcus with prontosil soluble renders the organisms more rapidly permeable by crystal violet, as shown by the rapidity of staining.

It has apparently been proved by Yorke *et al.* and Pedlow and Reiner that arsenical compounds and acriflavine, both of which are highly destructive for trypanosomes both in test tube and body, are rapidly fixed on these cells whether they be alive or dead. Strangely enough we have been unable to find any extensive proof that bactericidal substances like optochin, gentian violet and acriflavine are actually fixed on the microorganisms they affect, and diminished thereby in the supernatant fluid. One of us (Gay and Beckwith) began experiments of this sort some years ago, and they have now been extended in connection with the present investigation as a check on the mode of action of sulfanilamide.

In brief we find: 10 cc. of gentian violet (1–10,000) or the same volume of acriflavine (1–10,000) is reduced one-half in color index on addition of 1 cc. of a concentrated (× 100) culture of streptococcus, in from 1 to 3 hours. Correspondingly, the bactericidal titer is also reduced one-half. In the presence of rabbit serum (50 per cent) acriflavine is more active than in broth; it is impossible to measure the color reduction in the presence of serum but the bactericidal titer is reduced one-half, as in broth.

Turning now to contact between sulfanilamide and streptococci (100 cc. of an 18 hour broth culture reduced by centrifugalization to 1 cc.), we have never been able to show that the drug, 10 cc. (1–10,000 to 1–100,000), was reduced in strength in from 2 to 5 hours at room temperature. The Marshall colorimetric test for sulfanilamide was employed. Every variation in the conditions of this test that occurred to us was tried: growth of the streptococcus in 20 per cent serum broth with subsequent addition to sulfanilamide broth; growth of the streptococcus in broth with sulfanilamide for 18 hours, or in serum sulfanilamide broth for 10 hours.

Bürgers' contention was based on indefinite staining results. We

have grown streptococci in broth or in sulfanilamide broth 1–10,000 for 18 hours and found that the sulfanilamide broth-grown organisms, in aliquot numbers, adsorbed as much but no more gentian violet than the plain broth-grown organisms. Observationally there was no difference in the rapidity or intensity of staining of the organisms in the two preparations. Furthermore, neither sulfanilamide-grown organisms nor organisms in the presence of sulfanilamide were found to be more susceptible to the action of gentian violet and acriflavine than normal organisms.

The Effect of Sulfanilamide on the Virulence Factors of Streptococcus

Apart from growth inhibition and morphological changes wrought by sulfanilamide on the streptococcus, alteration and reduction in the virulence factors of streptococcus metabolism have been repeatedly claimed. Although the non-lethal effect of sulfanilamide on streptococcus may change temporarily the invasiveness of the organism it does not lead to any permanent impairment in its inherent virulence, as we pointed out and as has been confirmed by Lyons and Mangiaracine. After 2 or 3 days growth in sulfanilamide broth, our streptococcus H (adapted to rabbits), when inoculated intrapleurally in rabbits (M.L.D. ± 10 chains), or intraperitoneally in mice (M.L.D. ± 1000 chains), was just as virulent as the same organism grown in plain broth. In such experiments the scantier growth in sulfanilamide broth was adjusted to equal the number of organisms in the more luxuriant plain broth culture by dilution of the latter.

But the retention of potential pathogenicity does not mean that one or more of the virulence factors may not be temporarily inhibited in the presence of sulfanilamide. We have attempted to harmonize, for our own satisfaction, the somewhat discordant views that have been offered by numerous investigators on certain of the virulence factors, notably hemotoxin, leucocidin and fibrinolysin.

Hemotoxin Production.—It has been suggested as an hypothesis that the production of hemotoxin (hemolysin) may be inhibited when streptococci are in the presence of sulfanilamide or related compounds (Bürgers, 1938; Meyer, 1938), and some experimental work has been presented by Osgood, by Huntington, and by King, Henschel and Green to support this hypothesis. As an alternative explanation,

Levaditi and Vaisman, 1935 *b*; Meyer, 1937; and Gross, Cooper and Lewis, 1938 *a*, believed that although hemotoxin is formed it may be inactivated by the drug. There is evidence against this alternative hypothesis, in the hands of Kemp; Gross, Cooper and Lewis, 1938 *b*; Huntington; Osgood and Powell.

The following is a brief and general description of the methods used and the results obtained in our investigation of the effects of sulfanilamide upon hemotoxin produced by *Streptococcus haemolyticus in vitro*.

Organisms were grown either in broth and in broth plus sulfanilamide (concentrations varying from 1–1000 to 1–10,000), or in rabbit serum and rabbit serum plus sulfanilamide (same concentrations) for periods of 27 to 72 hours. This incubation period permitted the sulfanilamide to exert its modifying effects upon the organisms, and one of these effects was, invariably, a bacteriostasis. Because of this bacteriostatic effect, a plate count of the number of organisms in the control and in the sulfanilamide tubes was made, so that in the experiments equal numbers of organisms could be obtained by dilution of the control. Plate counts in the nineteen experiments indicated that growth of a large inoculum (*e.g.* 0.2 cc. of an 18 hour culture) of *Streptococcus haemolyticus* H, was always reduced in sulfanilamide media as compared with the control.

Organisms, then, which have been subjected for periods of 27 to 72 hours to the action of sulfanilamide were compared for hemotoxin production with their controls by means of the hemotoxin test described below:

Hemotoxin Test.—0.2 cc. (±1,000,000 streptococci), 0.1 cc. and 0.1 cc., of a 1–10 dilution, of the sulfanilamide-treated cultures were placed in separate Wassermann tubes. Amounts of untreated cultures diluted to correspond, in number of organisms, to the treated cultures were placed in other Wassermann tubes. To each tube 1 cc. of a 1 per cent suspension of washed rabbit red blood corpuscles was added, and the volume brought up to 2 cc. by addition of broth. The tubes were incubated at 37°C. and hemolysis noted at intervals by removing the tubes from the bath, centrifugalizing lightly to carry down the red cells, and comparing each tube in a comparator block with a series of standards. After each reading the tubes were shaken to resuspend the red cells, and immediately reincubated. The readings were continued until complete hemolysis was observed.

The results obtained are as follows: In every case of exposure of the organisms to the drug (whether in broth or in serum) the amount of hemolysis produced during the early hours of the test by the

treated organisms was less than that produced by the controls.   That this difference in hemolysis represents simply an inhibition in the ability to produce hemotoxin, or a delay only in its rate of production, is evident from the fact that in 15 of the 19 experiments at least one or more dilutions of culture of treated streptococci (6 to 7 hours) finally produced 100 per cent hemolysis of the red cells.

It seemed possible that the results obtained to this point might be due to the fact that the control organisms were growing more rapidly during the course of the test than those organisms which had been previously exposed to the drug.   To eliminate this possibility as a factor in the results, five experiments were performed in which (by means of frequent plate counts during the course of the test) a growth curve for the two cultures was obtained.   The growth curves of the control and treated cultures were essentially parallel.   In other words, the hemotoxin test was begun with equal numbers of untreated or sulfanilamide-treated organisms and as the test proceeded, both series grew out at the same rate.   A given number of the untreated streptococci produced hemolysis more rapidly than an equal number of sulfanilamide-treated streptococci.

In view of these results, the conclusion was reached that the difference in rate and amount of hemolysis between untreated controls and those exposed to the drug for 27 to 72 hours represents a definite though temporary alteration in the ability of the treated organisms to produce hemotoxin.

Experiments were also carried out to determine whether there was a decrease in the hemotoxin content of young streptococcus cultures in the presence of sulfanilamide.

Broth cultures were used in which the organisms were exposed to the action of the drug (concentrations of 1–1000 to 1–10,000) for periods varying from 3 to 15 hours.   In some cases the media were enriched by the addition of 2.5 per cent rabbit serum, in order to facilitate the production of hemotoxin.   The cultures were centrifugalized and the supernatant fluids placed in contact with rabbit red cells for 30 or 60 minutes.   Varying dilutions of supernatant fluids of both sulfanilamide broth and plain broth cultures were used, so that allowance might be made for the difference in the number of organisms in the two cultures.

Out of a series of tests, there was no indication that the sulfanilamide supernatant fluids contained less hemotoxin than the corresponding

control supernatant fluids.   The results of these experiments indicate that culturing streptococci in the presence of 1–1000 to 1–10,000 sulfanilamide for periods of 3 to 15 hours fails to alter the amount of hemotoxin produced by such treated cultures, and that the presence of the drug during the production of the toxin does not serve to neutralize it.

To determine whether sulfanilamide has any "neutralizing" action on hemotoxin already formed *in vitro* by untreated streptococci, the following experiments were carried out.   Both Berkefeld filtrates and centrifugalized supernatant fluid of a 6 hour broth culture were prepared.   To varying amounts (0.4 cc. to 0.9 cc.) of each, sulfanilamide in concentrations from 1–100 to 1–2000 was added.   Appropriate controls without sulfanilamide were prepared.   After one-half hour 1 cc. of a 1 per cent rabbit blood cell suspension was added and the time necessary for complete hemolysis noted.   The results indicated that sulfanilamide in concentrations varying from 1–1000 to 1–20,000 does not inhibit the hemolysis of a 1 per cent red cell suspension.   In other words, the drug does not neutralize or inactivate preformed hemotoxin present in the filtrate or supernatant of an untreated streptococcus culture.

Another type of experiment was conducted in an effort to check the results of early experiments of Osgood, who reported evidence of neutralization of hemotoxin by sulfanilamide as shown in blood agar plates. 1 cc. of a 1–5,000,000 dilution of an 18 hour streptococcus culture was added to blood agar containing sulfanilamide in concentrations varying from 1–5000 to 1–100,000, and pour plates made. Corresponding plates without drug were prepared.   One series of plates was incubated aerobically and another anaerobically.   The number of colonies on each plate was counted and the average diameter of the zone of hemolysis was determined by measuring 30 unselected colonies on each plate.   The colony count showed that the sulfanilamide was not bacteriostatic under these experimental conditions.   Measurements of the hemolytic zones indicated no decrease in the amount of hemolysis present in the sulfanilamide plates in either the aerobic or anaerobic series for any concentration of sulfanilamide used.   From these results it was concluded that sulfanilamide in blood agar pour plates, incubated aerobically or anaerobically, does not neutralize the hemotoxin formed, or check the growth of streptococcus colonies previously unexposed to the drug.

The final conclusions from our experiments, then, would be that residence of streptococci in sulfanilamide broth or serum may inhibit,

although only temporarily, their ability to produce hemotoxin. However, when the unaffected streptococcus is grown in the presence of varying amounts of sulfanilamide for short periods of time, no diminution in the hemotoxin formed can be noted. No evidence was found that sulfanilamide neutralizes hemotoxin already formed. In short, the supposed action of sulfanilamide on streptococcus hemotoxin can scarcely be used as explanatory of its therapeutic effect.

Leucocidin Production.—It has been suggested as an hypothesis without experimental proof that the therapeutic effect of sulfanilamide might be due to suppression of leucocidin formation, which property of streptococcus metabolism is known to be a factor in the virulence of this microorganism. The mere inhibition of growth might produce this result quantitatively (Mellon and Bambas; Bliss and Long). As corroborative evidence, leucocytes are relatively more normal in presence of sulfanilamide *in vitro* (Levaditi and Vaisman, 1935 *b*) or in marrow cultures (Osgood) than when the drug is absent.

We have attacked this problem more directly in the following manner:

Streptococcus was grown in plain broth, as control, or in broth plus sulfanilamide (from 1–1000 to 1–10,000). In ten different experiments, Berkefeld filtrates from such cultures or the supernatant fluids (at times heated to 56°C.) from centrifugalized cultures were used in varying amounts in order to equalize the difference in number of organisms in the two media. The Neisser-Wechsberg technique was employed (Gay and Oram).

In no instance was any difference in the leucocidin content, as measured by reduction of methylene blue, detected in the fluids of the sulfanilamide-grown cultures as compared with those of broth controls. Moreover, when direct observations were made on the appearance of the cells, in from 30 minutes to 2 hours, we could detect no difference in the degree of their injury in the two kinds of filtrates.

Fibrinolysin Production.—The species specific fibrinolysin formed by the hemolytic streptococcus is assuming an increasingly important significance in pathogenesis. Neither Huntington nor Kemp were able to demonstrate the inhibition of fibrinolysin in the presence of sulfanilamide experimentally.

In order to determine whether sulfanilamide interferes with the production or the activity of fibrinolysin, streptococcus was grown in broth plus sulfanilamide (1–10,000) or in broth without sulfanilamide for 24, 48 or 72 hours. At these intervals the cultures were plated to determine the relative number of living organisms. The Berkefeld filtrates from these cultures, used in varying amounts to equalize the differences in number of organisms, were tested for fibrinolytic activity, by the technique of Tillett and Garner. In all cases the filtrates of sulfanilamide broth cultures lysed the human clots at the same rate as did the filtrates from the plain broth cultures.

The fibrinolytic activity of the broth suspensions of the centrifugalized organisms from the 48 hour sulfanilamide broth cultures was also compared with that of the suspensions of the broth-grown organisms, diluted in order to compare equivalent number of organisms. The degree of lysis was noted at intervals and as soon as lysis of a clot was complete the number of organisms was determined by plating the contents of the tube.

The intervals of time for complete lysis of the clots by the organisms from sulfanilamide broth cultures were always longer than required for the lysis by the organisms from the broth cultures. These results, then, would appear to indicate that fibrinolysin is produced more slowly by the sulfanilamide-grown organisms, but the difference may be easily accounted for by the smaller number of sulfanilamide organisms at the time of complete lysis. The number of sulfanilamide broth-grown organisms was only one-third to one-tenth the number of broth-grown organisms when lysis was complete. When the fibrinolytic test was carried out with the control broth-grown organisms suspended in broth plus sulfanilamide (1–10,000) they produced lysis as rapidly as those resuspended in plain broth and the number of living organisms at that time was approximately the same in the corresponding tubes.

Thus, there is no inhibition in fibrinolysin production which is not directly referable to a difference in number of viable organisms.

The Indirect Effect of Sulfanilamide on Streptococcus

Sulfanilamide and related compounds that produce bacteriostasis of streptococcus have been shown by one of us with Warren and

Stokinger, to increase the normal oxidation-reduction potential in the growth curve of the microorganism when grown aerobically. It produces similar though less effect in broth alone. Other similar but non-bacteriostatic compounds (*e.g.*, prontosil) have no such action. It would seem that this change is in some way related to the effect of the drug on the streptococcus.

The suggestion of Main, Shinn and Mellon, and Locke, Main and Mellon that sulfanilamide may act as an anticatalase, thereby allowing unhampered production of peroxide, with harmful effect on the streptococcus, may also be a factor of importance. This does not account, however, for the fact that repeated doses of sulfanilamide fail to convert bacteriostasis to a true lethal effect.

Turning to another aspect of the indirect action of sulfanilamide, there is, and has been since the beginning of studies on this drug, evidence of its action simply as an adjuvant to the natural defense mechanism of the body. From the earliest studies the probable significance of mobile cell intervention has been mentioned (Domagk, 1935; Levaditi and Vaisman, 1935 *c*; Long and Bliss; Bürgers, 1937), and in later publications this was verified by more direct observations of macrophage phagocytosis by Levaditi and by Domagk, 1937.

The reticulo-endothelial system would not seem directly concerned with the rapid results obtained in chemotherapy by sulfanilamide (Levaditi and Vaisman, 1935 *c*; Gross, Cooper and Peebles). In our previous publication we also found no histological change in the fixed tissues which would account for the macrophage accumulation that, in our minds, accounts for the second and ultimately destructive effect *in vivo* that follows bacteriostasis by the drug. Although prolonged use of the drug does apparently lead to a reticulo-endothelial proliferation, in the hands of Davis *et al.*, this cannot, we believe, account for the immediate chemotherapeutic result. Sulfanilamide does not in itself attract leucocytes (Gay and Clark; Coman). There is no evidence that the microorganisms become drug-fast (Osgood). What the drug does do to the organisms is to render them temporarily impotent, and to reduce in slight degree their pathogenic properties. This inhibitory effect allows mobile cells, particularly the mononuclear cells of the connective tissue, the opportunity to accumulate, to approach, and to phagocyte the disabled bacteria. This accumu-

lation we have already demonstrated histologically in the pleural walls.

Our hypothesis as to the signal importance of host cells in sulfanilamide cure has hitherto been based largely on these histological studies of the tissues surrounding an area in which cure under the influence of sulfanilamide was taking place. The work as reported seemed to show a direct relationship between the disappearance of the enfeebled streptococcus and local mononuclear cell mobilization. We turn now to evidence tending to show that phagocytosis is more active with sulfanilamide-treated cocci or in the body of sulfanilamide-treated animals. For this approach we find more corroboration by other writers. Finkelstein and Birkeland found that guinea pig blood leucocytes phagocyted *in vitro* more streptococci in the presence of sulfanilamide in certain concentrations. Bürgers, 1938; Osgood; and Mellon and Bambas, however, found no evidence of any such alleged opsonic action. We have used leucocytes both from rabbit and guinea pig exudates and completely failed to confirm the results of Finkelstein and Birkeland.

We have approached this question experimentally in three different ways:

1. Sterile exudates were obtained from the pleural cavities of rabbits which had been inoculated with an aleuronat-starch mixture 24 or 48 hours previously. Some of the rabbits had received subcutaneously 4 to 7 injections of 10 or 20 cc. each of sulfanilamide (2 per cent). The injections were given from 24 or 48 hours until 3 to 4 hours before removal of the exudate. The controls were untreated. Streptococcus H (0.1 cc. or 0.2 cc. of an 18 hour broth culture) was added to the two kinds of pleural exudates (0.3 cc. to 0.8 cc.) and kept at 37°C., with constant agitation. Films were made at 1 and 2 hour intervals. At least 200 cells from each preparation were counted. In six experiments the amount of phagocytosis was practically the same in the exudates of the treated (average of phagocytic cells 4 per cent), as of the normal rabbits (average of phagocytic cells 3.5 per cent).

2. When immune serum was added to the exudates there was slightly more phagocytosis in the exudate of the sulfanilamide-treated rabbits (average phagocytic cells 8.5 per cent) than in the normal exudate (average phagocytic cells 6.5 per cent).

These experiments show clearly that no definite difference is observable in the *in vitro* phagocytosis by exudate cells from sulfanilamide-

treated rabbits, as compared with that of normal rabbits. The experiments, however, do not reproduce the actual conditions that obtain in therapeutic results in the body, in which latter the streptococci have been subjected over a longer period of time to successive doses of sulfanilamide.

Lyons and Mangiaracine have found that adult or infant human leucocytes, which have no effect on normal streptococci, do phagocyte streptococci that have been repeatedly subcultured in sulfanilamide. Our experiments with rabbit leucocytes agree with these results:

3. Leucocytes were obtained from the pleural exudate of 24, 48 and 72 hour aleuronat-starch prepared rabbits in six different experiments. Cultures were grown 24, 48 and 72 hours, either in plain broth or in broth containing sulfanilamide 1–10,000. Mixtures of whole exudate or its leucocytes in serum were made with treated and untreated streptococci. Tubes were agitated at 37°C. and counts made after ½ and 1 hour. Smears. 200 cells or more of each preparation were counted.

Whole exudate	Average of phagocytic cells per cent
Sulfanilamide broth streptococci	31.3
Broth streptococci	21
Leucocytes + normal rabbit serum, 56°C.	
Sulfanilamide broth streptococci	28.5
Broth streptococci	9
Leucocytes + rabbit immune serum	
Sulfanilamide broth streptococci	42.5
Broth streptococci	34.1

In all instances phagocytosis is greater when sulfanilamide-grown streptococci are put in contact with pleural exudate leucocytes, from the rabbit. This is true whether the phagocytic index is based on the percentage of all cells that are phagocytic or on the average number of chains of streptococci in each phagocytic cell.

When we turn to observations on the degree of phagocytosis in the animal body under treatment with sulfanilamide, several sets of experiments are already available. Levaditi and Vaisman, 1935 c, Bliss and Long, and Domagk, 1937, have all given evidence that the peritoneal exudate of sulfanilamide-treated mice shows greater phagocytosis of streptococci than the exudate of normal animals.

Our own experiments along this line, both in mice and in rabbits, also seem unequivocal.

1. Injection of Sulfanilamide-Grown or Normal Streptococci Intraperitoneally in Mice.—Streptococci grown for 48 hours, either in plain broth or 1–10,000 sulfanilamide broth, were centrifugalized and, after conservation at 0° while counts were made, were suspended in fresh broth so as to give the same number of organisms per cc. 0.5 or 1 cc. amounts of such suspensions were injected intraperitoneally in mice. The cavities had usually been prepared 2 to 3 hours before by broth injection. The exudates were examined in smears from $\frac{1}{2}$ to 6 hours later. After 4 hours the organisms were so numerous that accurate counts were impossible. Both types of culture produce a fatal infection.

In the earlier stages ($\frac{1}{2}$ to 1 hour) the average number of phagocytic cells containing sulfanilamide streptococci was 33.3 per cent, whereas only 20.3 per cent of cells contained the normal broth organisms. Counts made at later intervals showed much less difference, indirectly indicating that the sulfanilamide-treated organisms had regained their virulence.

2. Phagocytosis of Streptococci in Peritoneal Cavities of Mice Treated with Sulfanilamide, and of Normal Mice.—Mice were given intraperitoneal injections of 1 cc. (1–100) to 0.5 cc. (1–10) of an 18 hour broth culture of streptococcus H. This is approximately 1000 M.L.D. as this culture, adapted to rabbits, is relatively less virulent for mice. Cavities of a few were prepared 2 to 3 hours previously with broth. Half of the mice were treated subcutaneously with 0.5 cc. of 2 per cent sulfanilamide and the others left as controls. The majority of the treated animals were given the drug 2 and 4 hours after infection, but a few received it simultaneously or a few hours previously. Observations and counts were made on the exudates removed at hourly intervals for from 2 to 8 hours. Observations on 7 treated animals and 6 controls are included. 5 controls were dead before 24 hours, and 1 treated mouse died in 48 hours.

The average counts of phagocytic cells on the entire range from 2 to 8 hours do not show any higher percentage of phagocytosis in the sulfanilamide-treated mice than in the controls. When the counts of the later periods only (6 to 8 hours) were averaged, there was a slight difference shown, 10.8 per cent for the sulfanilamide-treated mice, and 7 per cent for the control mice.

3. Phagocytosis in Pleural Cavities of Rabbits Treated with Sulfanilamide as Compared with That in Cavities of Normal Rabbits.—Rabbit pleural cavities were prepared either 24 or 48 hours previously with aleuronat-starch, or 3 hours previously with broth, and injected intrapleurally with concentrated fresh broth-grown cultures of streptococcus H (approximately 250 million). Half of these rabbits (total 4) were given injections of sulfanilamide subcutaneously; the first 1 to 3 hours before and a second simultaneously, or 1 hour after the injection of streptococcus. The exudates were examined 2 to 4 hours after infection. There was a control rabbit in each of the four experiments tried.

The average percentage of phagocytic cells in the sulfanilamide-treated rabbit was 14 per cent, as contrasted with 5 per cent phagocytic cells in the contro rabbits.

It is difficult in experiments of this type, and particularly with th strain we have employed, to obtain conditions of treatment by sul fanilamide that will result in cure, and at the same time guarantee th presence of sufficient numbers of streptococci to afford good estimate: of the degree of phagocytosis. In our mouse experiments, sulfanil amide-grown streptococci are at first better phagocyted *in vivo* thar are normal cocci. In the experiments with sulfanilamide-treatec mice, although a curative dose is used, superior phagocytosis is not clearly demonstrable.

Our experiments in the rabbit pleural cavity would seem clearly to indicate that even when enormous multiples of the lethal dose are employed (to give phagocytic pictures) phagocytosis is definitely superior in sulfanilamide-treated animals, as contrasted with untreated rabbits.

CONCLUSIONS

The precise mode of therapeutic action of sulfanilamide on strepto-coccus can be arrived at only by considering the sum total of factors that inhibit or favor the natural growth of the microorganism under the experimental conditions that obtain, whether *in vivo* or *in vitro*. Too sweeping conclusions have hitherto been drawn from the study of a single variable factor, such as an unfavorable temperature or the absence or presence of peptone. We have attempted here to analyze the factors that have hitherto been recognized and some new ones, but particularly the relationship of these factors to one another.

The result obtained on adding sulfanilamide to the streptococcus in the test tube is usually bacteriostasis and not complete destruction of even small numbers of bacteria. This is on the condition that the suspending medium is a favorable one for the growth of the micro-organism; the more growth-promoting the medium is the less the bacteriostasis. If, on the other hand, the medium is too poor, or one that in itself inhibits growth, the addition of sulfanilamide may lead to sterilization of the culture.

The conditions for growth of the streptococcus in the body of the rabbit or mouse, depend on the strain of bacteria used, but are on the whole favorable. Defence, however, in the form of phagocytosis by both polymorphonuclear and by mononuclear cells is attempted even in the susceptible animal. When sulfanilamide is used to treat such an animal, or when sulfanilamide-grown (inhibited) streptococci are employed, phagocytosis is pronounced, whether studied in the test tube or in the animal body. In the rabbit the delay by sulfanilamide and resultant increased phagocytosis by polymorphonuclears allows mononuclear cells to accumulate and recovery may result.

Sulfanilamide not only does not completely destroy the streptococcus but does not even impair its innate virulence. It acts upon the streptococcus not only by inhibiting growth but by a temporary inhibition of hemotoxin formation, but only under certain conditions. The drug does not neutralize hemotoxin already formed. No significant effect of sulfanilamide on the formation of leucocidin or fibrinolysin by streptococcus has been evident in our experiments.

Sulfanilamide differs in one important respect from other drugs that are destructive either in the test tube or actually in the body, for protozoa and bacteria. Protozoa fix or adsorb arsenicals and acriflavine that kill them variably *in vitro* and *in vivo*. Streptococci fix both gentian violet and acriflavine, which dyes have marked destructive action in the test tube but are less effective *in vivo*. Sulfanilamide is not diminished at all by contact *in vitro* with large masses of streptococci, nor does the action of this drug render the microorganism more capable than untreated cocci to adsorb gentian violet or acriflavine, or to be destroyed by these highly bactericidal substances.

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CAUSES OF THE CESSATION OF GROWTH OF FIBROBLASTS CULTIVATED IN EMBRYO JUICE*

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PLATE 37

Fibroblasts cultivated according to the flask technique in a plasma coagulum with embryo juice as nutrient fluid do not proliferate continuously. Generally, after 2 or $2\frac{1}{2}$ weeks of cultivation the cells stop multiplying although only a small portion of the coagulum is covered with tissue. If a part of the tissue is transferred to a new coagulum, growth is resumed. If it is not so transferred, the cells degenerate. Investigators who have attempted to find the cause of this cessation of growth and subsequent degeneration of the cells, have generally attributed it either to changes that occur in the physical structures of the coagulum, or to the accumulation of toxic products therein.[1] Some of these investigators have cut out a section of the old coagulum in which fibroblasts had stopped growing and have filled in the aperture with fresh plasma. The renewed growth that resulted was taken as evidence that the cells were able to invade a newly formed coagulum but did not have the power to invade one that had aged.[2] Acting on this hypothesis, Mayer has developed a technique for growing large colonies of fibroblasts by periodically cutting away all the old coagulum surrounding the colony of cells and then building a new coagulum around them. Embryo juice is supplied, of course, as the nutrient. In this way he has obtained cell colonies $2\frac{1}{2}$ cm. in diameter.[3] But in

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* Reported in brief in *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 369.

[1] Ephrussi, B., *Arch. anat. micr.*, 1933, **29**, 95. Fischer, A., *Cytologia*, 1930, **1**, 217. *Virchows Arch. path. Anat.*, 1930, **279**, 94. Mayer, E., *Arch. Entwcklngsmcchn. Organ.*, 1933, **130**, 382; *Compt. rend. Soc. biol.*, 1935, **119**, 422. Olivo, O. M., *Arch. exp. Zellforsch.*, 1931, **11**, 272.

[2] Ephrussi, B., *Arch. anat. micr.*, 1933, **29**, 95. Fischer, A., *Cytologia*, 1930, **1**, 217.

[3] Mayer, E., *Compt. rend. Soc. biol.*, 1935, **119**, 422.

all of this work no attention has been paid to the nutritive value of the serum that is gradually removed from the coagulum as cultivation is continued, and is present again when a new coagulum is formed. The purpose of the present investigation was to ascertain if it is not the removal of this serum, rather than the aging of the coagulum, that is responsible for the cessation of growth. Or, to express it in another way, to ascertain if the cessation of growth were not due to an inadequacy in the food supplied.

Preliminary Experiment

To test this hypothesis, two fragments of chick heart fibroblasts from a 23 year old strain were embedded in a Carrel flask in a coagulum containing 33 per cent plasma, and cultivated for 9 days in 33 per cent embryo juice.[4] By this time their initially rapid growth had already decreased to a noticeable degree.[5] 2 drops of chicken serum were then given every 2 days in addition to the embryo juice that had previously been supplied. Active growth was immediately resumed. However, after 9 more days of cultivation, it was evident that the rate of growth was decreasing again. Since the colonies had now become quite large it seemed that this decrease in growth might be due to an insufficient amount of food rather than to an inadequacy in the nature of the food given. Therefore, the concentration of the embryo juice in the medium was increased from 33 to 66 per cent, and the serum was given as before. Again, active growth was resumed; and this time it continued until the edge of one of the colonies reached the vertical side of the flask. A growth curve of one of these colonies showing the changes in rate of growth with each change in the medium is reproduced in Text-fig. 1.

These results indicate that two changes in procedure are required to produce continued growth of heart fibroblasts; first, that serum as well as embryo juice should be given, and second, that the embryo juice should be supplied in higher concentration than has previously been thought desirable. The experiments that follow were designed to test each of these hypotheses in as thorough a manner as possible.

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[4] The embryo juice was prepared by extracting one volume of the latapie pulp of 9 or 10 day old embryos with 3 volumes of Tyrode's solution.

[5] The coagulum in this experiment was made of horse plasma. This accounts for the decrease in the rate of growth sooner than it is usually observed with tissues embedded in coagula made of chicken plasma.
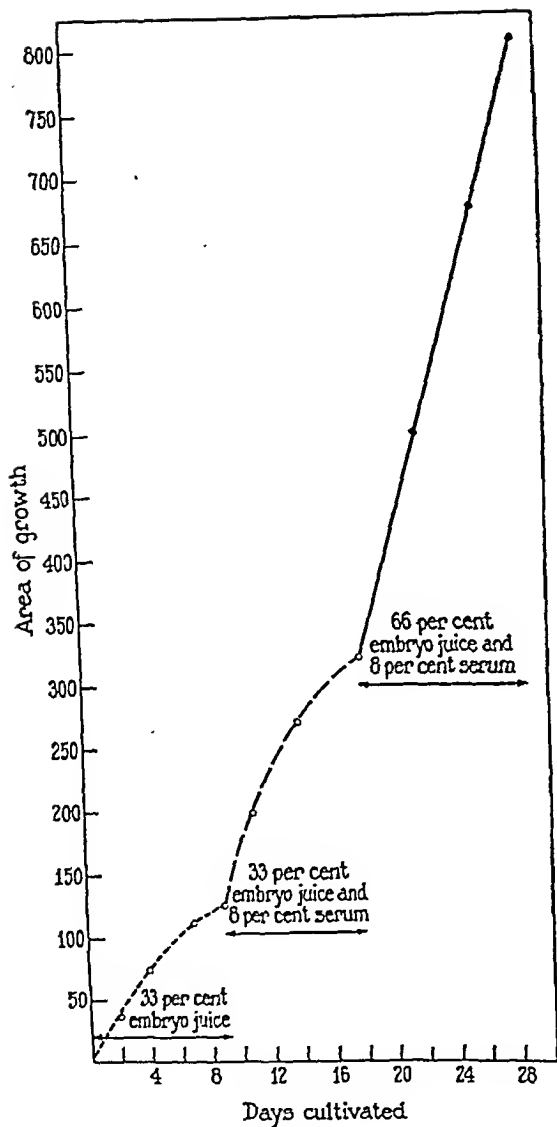
TEXT-FIG. 1.

TEXT-FIG. 2.

TEXT-FIG. 1. Experiment 15565-D. Growth curve of chick heart fibroblast culture from a 23 year old strain which was cultivated for 9 days in 33 per cent embryo juice, then for 9 days in 33 per cent embryo juice and 8 per cent chicken serum, and finally for 11 days in 66 per cent embryo juice and 8 per cent serum. Two tissues in a flask. Coagulum made of citrated horse plasma.
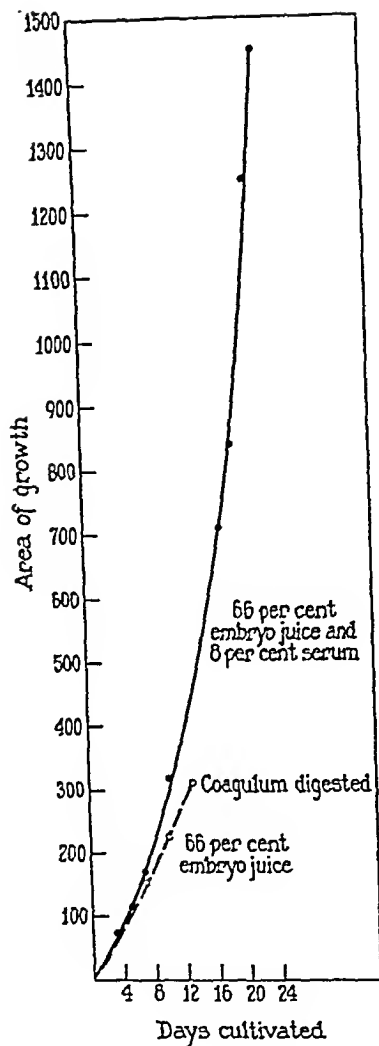
TEXT-FIG. 2. Experiment 15753-D. Comparison of extent and duration of growth of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice supplemented with 8 per cent chicken serum. A single fragment of tissue in each flask. Coagula made of chicken plasma.

Confirmatory Experiments

The confirmatory experiments that were made may be divided into three groups. Those in the first group were designed to test the extent and duration of growth of sister colonies of fibroblasts when one was given embryo juice alone in the nutrient fluid, the other a mixture of embryo juice and serum. 66 per cent embryo juice was supplied to both cultures in some of these experiments, 33 per cent in others. The concentration of serum was varied from 4 to 8 per cent. In the second group of experiments the extent and duration of growth of sister colonies of fibroblasts was compared when one was cultivated in 33 per cent embryo juice, the other in 66 per cent embryo juice. In some of these experiments no serum was given to either culture. In others, serum was supplied to both. The third group of experiments was designed to ascertain whether the serum was needed mainly as a nutrient or functioned merely to preserve the coagulum. In the first of these experiments embryo juice at 66 per cent concentration was given to both cultures. One of them received 4 per cent serum in addition, the other 8 per cent serum. Then sister colonies of fibroblasts were cultivated without a coagulum, one in embryo juice alone, the other in a mixture of embryo juice and serum.

Procedure Followed in the Confirmatory Experiments

All the experiments were performed first with chick heart fibroblasts from a 23 year old strain. Then a number of them were repeated with chick heart fibroblasts from a new strain that had been cultivated only six passages *in vitro* before it was taken for these experiments.[6] Each culture was divided into two equal parts. These were cultivated in separate Carrel flasks 3⅓ cm. in diameter. With the exception of the few experiments in which a fluid medium was used, they were embedded in coagula 1.2 cc. in volume, containing 33 per cent plasma, and embryo juice at whatever concentration was to be used in the medium. Homologous plasma was used in some of the experiments, heterologous plasma (citrated horse or citrated irradiated cow plasma) in others. Coagulation of the citrated plasma was brought about by the addition of calcium-Ringer's solution prepared as described by Vogelaar and Erlichman.[7] Unless otherwise specified, a single

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[6] A few experiments were also made with fresh heart tissue taken directly from the embryo. A longer time was required to obtain the results with the fresh tissue since it contained enough stored nutriment to maintain the cells for some time.

[7] Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer*, 1933, **18**, 28.

fragment of tissue was placed in each flask as near the center of the coagulum as possible. When the coagula were made with homologous plasma, no serum was given during the first week of cultivation since that already in the coagulum sufficed during this time. Phenol red at 0.005 per cent concentration was incorporated in all media to serve as an indicator of pH, and the acidity was adjusted by using a gas mixture containing 3 per cent CO_2, 21 per cent O_2, and 76 per cent N_2. Every 2 days the cultures were washed for 2 hours at 37°C. with $1\frac{1}{2}$ cc. of their respective media. Then this wash fluid was withdrawn and the cultures were returned to the incubator. Whenever the cell colonies became very large, new medium was given every 24 hours instead of every 48 hours, and 0.5 cc. of medium was placed on the culture after the wash fluid had been withdrawn. The embryo juice was generally made by extracting for $\frac{1}{2}$ hour one volume of pulp from 9 or 10 day old chick embryos with three volumes of Tyrode's solution. In some instances the embryo juice was made with glucosol solution. Then Tyrode's solution containing twice the usual amount of bicarbonate was used to complete the media. The serum was taken in each instance from adult cocks.

Comparison of Growth in Embryo Juice with and without Serum

In the experiments of group I, in which the tissues were embedded in homologous plasma and embryo juice was supplied at 66 per cent concentration, the sister colonies, *i.e.*, those given embryo juice alone and those given serum with the embryo juice, proliferated very rapidly and at approximately the same rate for 11 or 12 days. Then the colonies that were being cultivated in embryo juice alone suddenly liquefied the coagula. Those that were given serum with the embryo juice continued their active proliferation until the entire coagulum was covered with tissue (Text-fig. 2). Thus, colonies that were 3 to $3\frac{1}{2}$ cm. in diameter were obtained. The time required for the cells to cover the coagulum varied with the original condition of the tissue. The new strain of fibroblasts that had been cultivated only six passages *in vitro* before it was taken for this experiment proliferated so rapidly that the cells from a single fragment of tissue covered the coagulum in 17 days. Single fragments of tissue from the 23 year old strain required from 25 to 36 days in the different experiments.

In an attempt to circumvent digestion of the coagulum by the tissue cultivated in embryo juice alone the experiments described above were repeated, using heterologous plasma in making the coagula. Under these conditions liquefaction of the clot was delayed but not prevented. However, before the coagula were digested a marked difference in the

rate of growth of the colonies cultivated with and without serum was noted. Those that received serum with the embryo juice increased in area and also in density more rapidly than those cultivated in embryo juice alone. While the colonies cultivated in embryo juice alone proliferated mainly at the periphery of the culture, those culti-
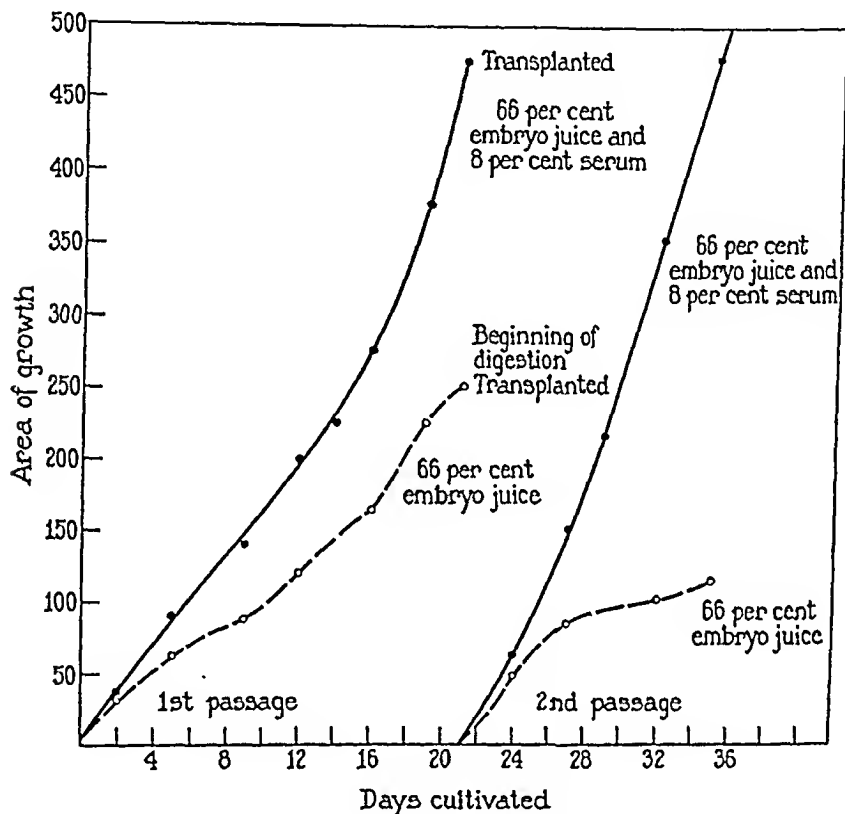
TEXT-FIG. 3. Experiment 15477-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain embedded in coagula made of irradiated, citrated cow plasma, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice and 8 per cent chicken serum. Two fragments in a flask. The tissues were transplanted into new coagula on the 21st day because those in embryo juice alone were beginning to digest the coagulum.

vated in the mixture of serum and embryo juice proliferated to a considerable extent at the center as well, and formed colonies containing many layers of cells. After 2 or 3 weeks of cultivation the colonies that were given embryo juice alone liquefied the coagula. Those cultivated in embryo juice and serum proliferated as long as

cultivation was continued. Curves illustrating the difference in rate of growth in one of these experiments, in which the tissues were transplanted when those in embryo juice alone began to digest the coagulum, are reproduced in Text-fig. 3.

To see if the difference in the rate of growth observed in the heterologous plasma could be observed in homologous plasma also, the experiments were made once more in coagula of homologous plasma. But this time, the clots were washed as soon as coagulation had occurred so as to remove a considerable part of the serum they contained. Under these conditions the same difference in growth was observed as had been noted in the heterologous plasma (Text-fig. 4).

The results obtained in 33 per cent embryo juice differed considerably from those just described. The control colonies, *i.e.*, those given embryo juice alone as nutrient fluid, behaved differently in different experiments. Usually they proliferated rapidly at first, then more slowly, and finally not at all. Soon after their proliferation ceased, the cells degenerated. The growth curve of these colonies was the typical S-shaped curve that has frequently been described.[2] However, in a few instances, growth continued 28 days or longer (Text-figs. 5 and 6). Therefore, colonies of large area were formed, but the cells in those colonies were scattered and the tissue was exceedingly thin. It was obvious, moreover, that the cells within these colonies were gradually digesting the coagula, and obtaining additional nutriment thereby. The coagula became thinner and thinner, and in certain instances appeared to be consumed in that region that was covered with cells. When serum was given with the embryo juice, the coagula retained their original thickness for a long time. Moreover, the tissue formed in the mixture of embryo juice and serum was always more dense, and was composed of a larger number of cell layers than was that formed by the sister colony cultivated in embryo juice without serum (Figs. 1, 2, and 3). When 8 per cent serum was given with 33 per cent embryo juice the cells became quite granular, and the colony cultivated in embryo juice and serum increased in area less rapidly than the sister colony that was given only embryo juice in the nutrient fluid. But since the growth continued for a longer time when serum was present, the colonies in the mixture of embryo juice and serum eventually outgrew those given embryo juice alone (Text-
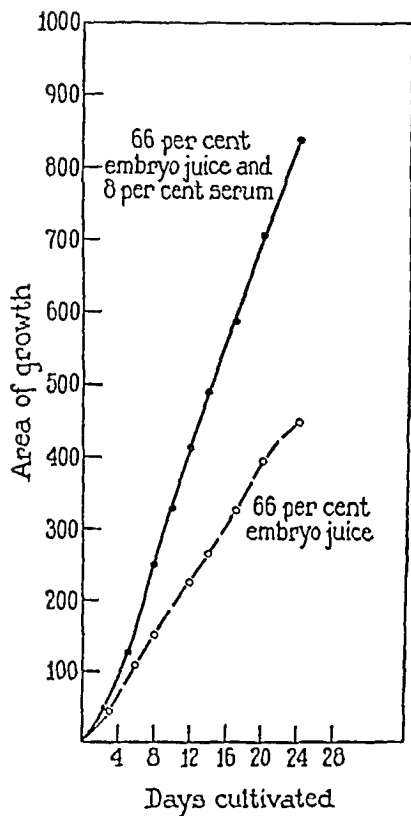
TEXT-FIG. 4

TEXT-FIG. 5

TEXT-FIG. 4. Experiment 15747-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice and 8 per cent serum. Coagula made of chicken plasma and then washed to remove a part of the serum.
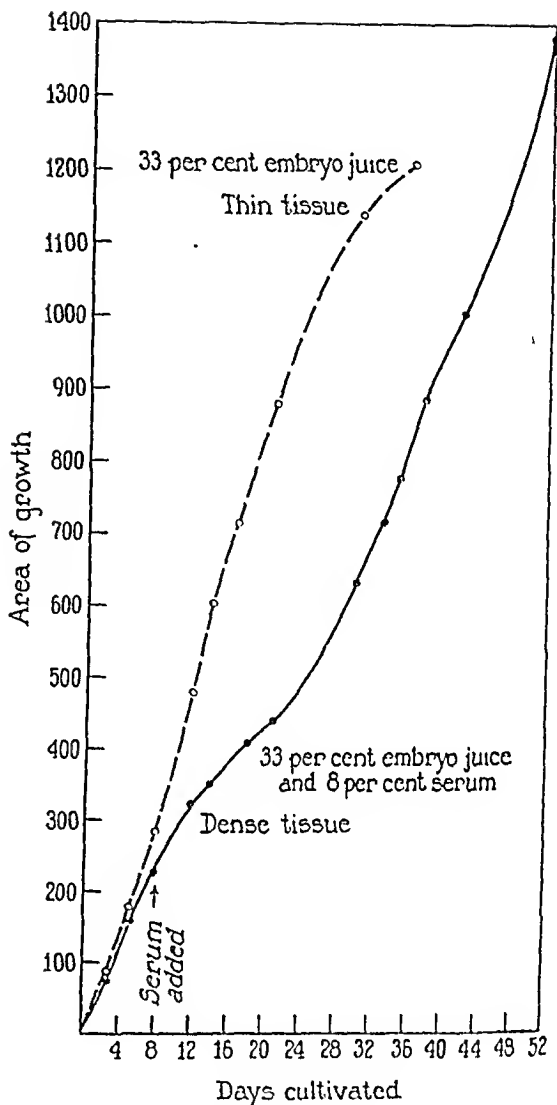
TEXT-FIG. 5. Experiment 15826-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 33 per cent embryo juice, the other in 33 per cent embryo juice supplemented with 8 per cent serum. Coagula made of chicken plasma. One fragment of tissue in a flask. The colony in embryo juice alone consisted of a very thin growth of rather scattered cells. The other formed a dense tissue. The former derived additional nourishment by slow digestion of the coagulum.

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fig. 5). When 4 per cent serum was given with 33 per cent embryo juice, the cells remained in excellent condition; and the colony which received the serum increased in area and also in thickness more rapidly than did the sister colony that was cultivated in embryo juice without serum (Text-fig. 6). But growth in this medium was not continuous. After colonies 2 or $2\frac{1}{2}$ cm. in diameter were obtained, proliferation ceased. Yet the cells cultivated in the mixture of 33 per cent embryo juice and 4 per cent serum did not degenerate, as those in embryo juice alone did. Some of them were kept under cultivation for 2 and 3 months. Throughout this time they seemed to be in good condition. Then, when a small fragment of the tissue was transplanted, they proliferated again. It seems, therefore, that an equilibrium is reached in this medium in which the food supplied is sufficient to maintain a large colony of cells, but is not sufficient to promote further proliferation.

Comparison of Growth in 33 Per Cent and 66 Per Cent Embryo Juice, with Serum, and without Serum

In all those experiments in which the growth in 66 per cent embryo juice was compared with that in 33 per cent embryo juice, a much more rapid growth was always obtained at the higher concentration. When no serum was supplied with the embryo juice the colonies that were given embryo juice at 66 per cent concentration always liquefied the coagulum. Those cultivated in 33 per cent embryo juice proliferated actively at first then more slowly, and then as a rule degenerated without digesting the coagulum. When serum was given with the embryo juice the colonies that received embryo juice at 66 per cent concentration proliferated until the entire coagulum was covered with tissue. Those given embryo juice at 33 per cent concentration proliferated for a very long time but never filled the flask (Text-fig. 7).

Cultivation with and without a Coagulum in Media Containing Different Amounts of Serum

So much has been said concerning the probable effect of changes in the physical structure of the coagulum on growth that it seemed advisable to establish, if possible, whether the growth-promoting effect
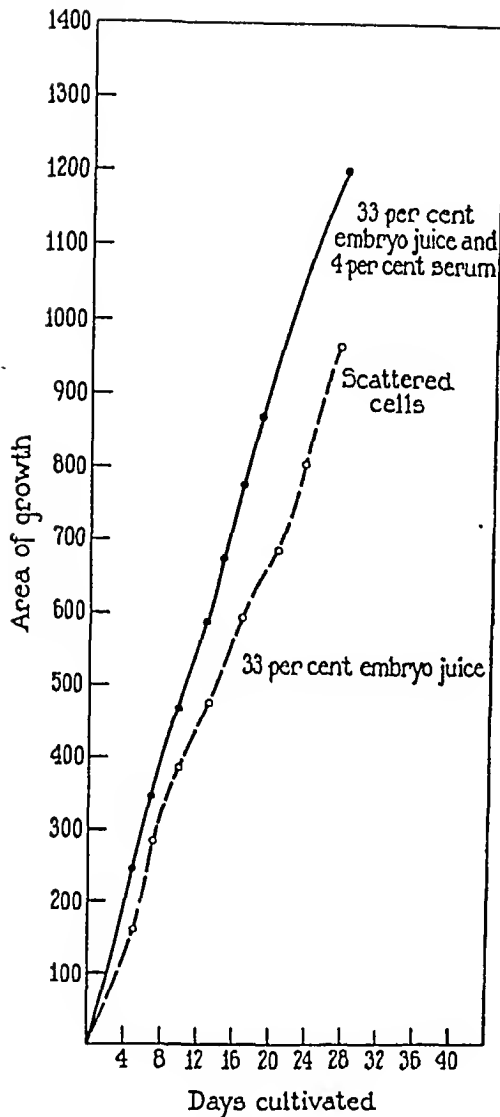
TEXT-FIG. 6

TEXT-FIG. 7

TEXT-FIG. 6. Experiment 15811-D_{1+2}. Growth curves of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one of which was cultivated in 33 per cent embryo juice, the other in 33 per cent embryo juice supplemented with 4 per cent serum. A single fragment of tissue in each flask. Coagula made of chicken plasma. The tissue in embryo juice alone was not as dense as the other, and it digested its coagula at a very slow rate.
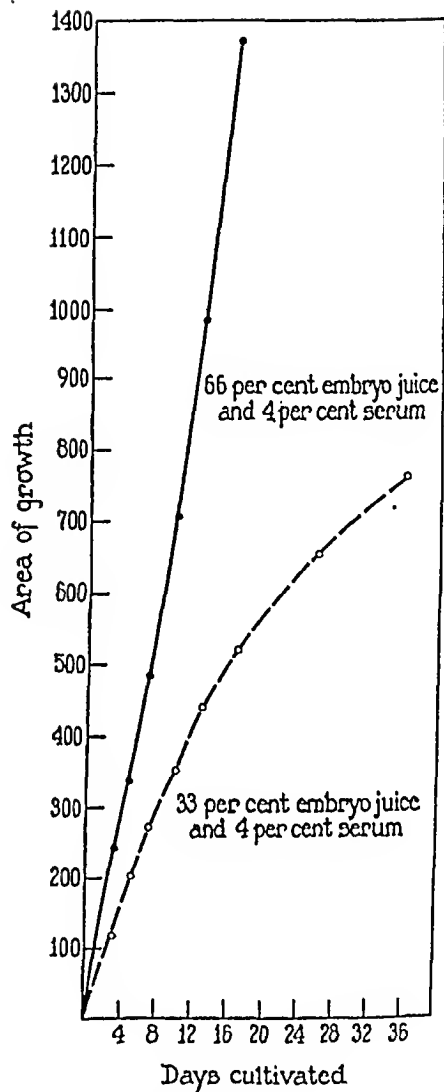
TEXT-FIG. 7. Experiment 17811-D_{7+8}. Growth curves of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one of which was cultivated in 33 per cent embryo juice supplemented with 4 per cent serum, the other in 66 per cent embryo juice supplemented with the same amount of serum. A single tissue in each flask. Coagula made of chicken plasma.

of the serum, described under the experiments of group I, was the direct effect of nutrients in the serum, or the indirect effect of its
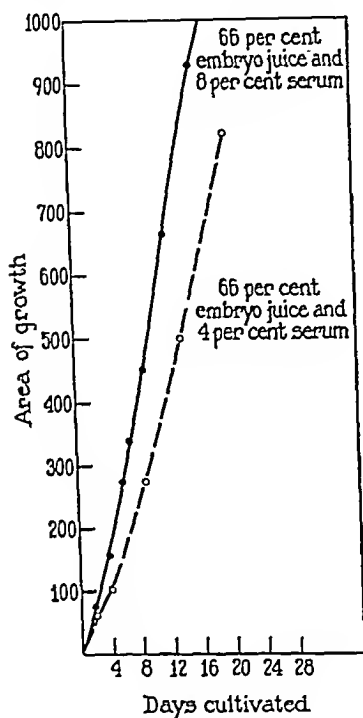
TEXT-FIG. 8.

TEXT-FIG. 9.

TEXT-FIG. 8. Experiment 15911-D. Growth curves of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 66 per cent embryo juice and 4 per cent serum, the other in 66 per cent embryo juice and 8 per cent serum.
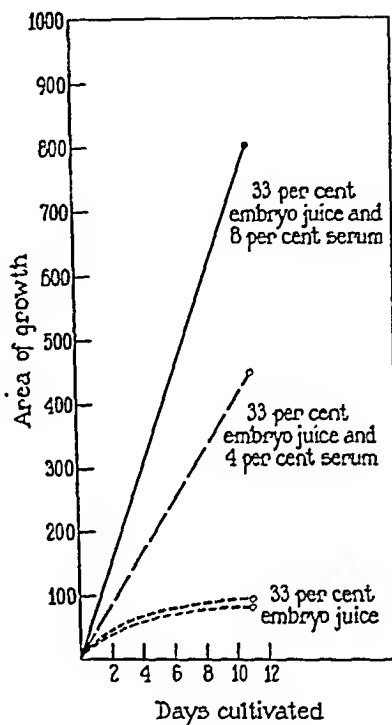
TEXT-FIG. 9. Experiment 15818-D. Areas attained in 11 days by four sister colonies of chick-heart fibroblasts in their seventh passage *in vitro*, when cultivated in fluid medium, two of them in 33 per cent embryo juice, one in 33 per cent embryo juice and 4 per cent serum, and one in 33 per cent embryo juice and 8 per cent serum.

preservative action on the coagulum. In the first experiment made to throw some light on this question, sister colonies of fibroblasts were cultivated in 66 per cent embryo juice. One of them was given 4

per cent serum in addition, the other 8 per cent serum. The coagulum was preserved in both instances, but greater proliferation was obtained with 8 per cent serum (Text-fig. 8). In the second experiment the tissues were cultivated in fluid media, in embryo juice alone, and in a mixture of embryo juice and serum. Cells from the colonies cultivated in embryo juice alone migrated out onto the glass, but the tissue that was formed broke up almost immediately into islands of a few cells each, then into isolated cells which soon died (Figs. 4 and 5). Those cultivated in the mixture of embryo juice and serum proliferated much more actively. The cells migrated out onto the glass and formed a tissue of connected cells which showed no tendency to break up into isolated cells. Then groups of cells migrated further out, forming islands of tissue that covered the entire flask[8, 9] (Figs. 6 and 7). In these experiments as in those just described, a marked difference in the rate of growth was noted according to the amount of serum supplied, that in the medium containing 8 per cent serum being greater than that in the medium containing only 4 per cent (Text-fig. 9).

DISCUSSION

It is evident from the results described above that the primary cause of the discontinuance of growth of heart fibroblasts when they are cultivated in a plasma coagulum with embryo juice as nutrient fluid is the removal of serum from the coagulum. When serum as well as embryo juice is supplied in the nutrient fluid the cells proliferate more actively and for a longer time than they do in embryo juice alone. Yet active growth does not continue unless the embryo juice is given

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[8] A strikingly different result was obtained with fresh heart tissue in fluid medium. Fairly large colonies of fibroblasts were obtained in embryo juice alone, and these adhered to the flask for a much longer time than did the tissue cells from the pure strain. No attempt has been made to ascertain for how long a time the tissue could be cultivated under these conditions.

[9] Fibroblasts have been cultivated by des Ligneris for several months in hanging drop cultures in a medium prepared by extracting embryo pulp with serum. No attempt was made in that work to ascertain the relative part played by the two constituents of the medium. The precipitate that was formed in the medium on standing also appeared in this work and eventually obscured the outline of the cells (des Ligneris, M. J. A., *Arch. exp. Zellforsch.*, 1936, 18, 442).

at higher concentration than that usually supplied. The question of promoting continuous growth seems, therefore, to be mainly a question of supplying adequate nourishment. A means must be found, of course, of bringing the nourishment into intimate contact with the cells, and of removing the waste products. The efficiency with which this is done assumes ever greater importance as the mass of the tissue increases. The serum functions in two ways. It supplies nutriment that is needed by the cells and prevents digestion of the coagulum. Sometimes in its absence the cells digest the coagulum at a very slow rate and obtain thereby enough nutriment to enable them to live for a considerable period of time, but their proliferation under these conditions is never as rapid as it is when serum is present.

That serum is a valuable nutrient for fibroblasts has been known for a long time. Carrel in his first experiments with tissues cultivated outside the body maintained heart and other embryonic tissues in plasma and serum for 2 months or longer.[10] Olivo in 1931 cultivated a fragment of embryonic heart for 6 months in diluted plasma and serum;[11] and Parker maintained fibroblasts from various sources in diluted serum for periods extending from 92 days to an entire year.[12] But in all of these experiments growth was exceedingly slow. In Carrel's experiments the fragments grew smaller and smaller, a part of the tissue being lost at each transfer. Olivo reports that in 6 months cultivation the fragment only doubled its size; and Parker states that while serum promotes a slow growth, the first effect of the serum is invariably injurious. The degree of injury, and also the ability of the cells to utilize the serum and proliferate in it varies with the different strains, i.e., according to the source from which they are derived. Fibroblasts from heart utilized the serum less readily than did the other strains and suffered greater injury at first.[12] Moreover, in Parker's experiments the muscle fibroblasts which he cultivated for an entire year grew so slowly that it was not necessary to transfer them to a new flask throughout that time. The marginal cells of such

[10] Carrel, A., *J. Am. Med. Assn.*, 1911, 57, 1611; *J. Exp. Med.*, 1912, 15, 516.
[11] Olivo, O. M., *Arch. exp. Zellforsch.*, 1931, 11, 272.
[12] Parker, R. C., *J. Exp. Med.*, 1933, 58, 97; 1936, 64, 121.

cultures invariably died, although new ones came from the central fragment at periodic intervals.

Earle has reported that horse serum is a satisfactory medium for mammary carcinoma and normal fibroblasts taken from the subcutaneous tissues of the rat, and that the growth of these tissues in horse serum is increased by the addition of embryo juice. He also records in passing that the fibroblasts grew more actively in this mixture than in any he had tried.[13] Lewis, and Gey and Gey have found mixtures of human cord serum, rat serum, and embryo juice the most suitable medium for sustaining growth of various tissues in their roller tube technique.[14] Yet the idea that heart fibroblasts require only embryo juice as a nutrient still persists. Moreover, none of these workers have attempted to show by comparative tests the part played by the serum in producing and maintaining growth. Therefore, although some of the results reported[15] here are implied in some of this work, it has seemed advisable to report these comparative experiments which demonstrate that even that strain of fibroblasts which utilizes serum the least readily of all the fibroblasts, when serum alone is given, actually requires serum for its growth.

No effort has been made in this work to determine the optimum ratio of serum to embryo juice or the exact composition of a medium that would promote maximum growth. It is evident, nevertheless, that the concentration of serum should be varied with that of the embryo juice.

It is probably important to note that these experiments do not necessarily invalidate those reported many years ago by Carrel and his

[13] Earle, W. R., *Am. J. Cancer*, 1935, 24, 567.

[14] Lewis, W. H., *Carnegie Institution of Washington, Pub. No. 150, Contrib. Embryol.*, 1935. Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, 27, 45.

[15] The experiments reported here were actually performed in 1933 and 1934. Pressure of other work being done at the time prevented their immediate publication. Then this other work which implied these results appeared, so publication did not seem essential. Yet because these experiments show better than others the real part played by the serum, and because of the persistence of the idea that serum inhibits the growth of heart fibroblasts, it has seemed advisable to present them at this time.

coworkers[16] on the inhibiting effect of serum when it is used with embryo juice. In those old experiments serum was always used at high concentration, usually as ½ or ⅔ of the medium; while here it was used at 8 per cent concentration or less.[17] In a few of these experiments, in which the embryo juice was used at 33 per cent concentration and serum was given at 8 per cent concentration,[17] the effect which serum had in restricting the area of migration was very evident. Moreover, the cells became very fatty and granular when the ratio of serum to embryo juice was too high. Since serum is antitryptic in its action, and since the embryo juice is enzymatic, it may well be that a high concentration of serum prevents growth by inhibiting enzymatic action, while a low concentration promotes it. It is also important to note that these experiments do not necessarily mean that it is impossible to prepare from embryos alone a medium that will meet all the nutritive requirements of the fibroblast; but until the continuous proliferation of fibroblasts in embryo juice alone has been found possible, it will be well to consider serum as an essential part of the medium.

The findings reported here apply only to heart fibroblasts since it is only heart fibroblasts that have been used in this work. In all probability the same will be found true of fibroblasts from other sources. However, it is not advisable to assume that such is the case for all fibroblasts until it has been submitted to experimental test, for as Parker has shown, fibroblasts of different origins have different nutritional requirements.

SUMMARY

Experiments designed to ascertain the reason for the cessation of growth of heart fibroblasts when they are cultivated in a plasma coagulum with embryo juice as nutrient fluid have shown that it is due, first, to the gradual removal of serum from the coagulum, and

[16] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, 34, 317, 599; 1922, 35, 17, 647; 36, 399; 1923, 37, 759. Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, 42, 143; 1927, 45, 305.

[17] Actually the concentration of serum was somewhat higher than this since there was also some serum in the coagulum.

second, to an insufficient supply of embryo juice. In a medium containing embryo extract at 66 per cent concentration and serum at 8 per cent concentration, growth continued until the entire coagulum in a $3\frac{1}{2}$ cm. flask was covered with tissue. The serum is needed to furnish additional nutriment, and also to prevent digestion of the coagulum.

EXPLANATION OF PLATE 37

FIGS. 1 and 2. Photographs showing the relative density of sister colonies of fibroblasts cultivated in embryo juice with and without serum. Fig. 1, fibroblasts near the periphery of a colony, cultivated for 36 days in 33 per cent embryo juice; Fig. 2, fibroblasts in a corresponding area of a sister colony, cultivated for 36 days in 33 per cent embryo juice and 4 per cent serum. $\times$ 115.

FIG. 3. Same colony as shown in Fig. 2. An area near the center of the colony. $\times$ 115.

FIG. 4. Cells that have migrated out onto the glass from colonies of chick heart fibroblasts from a 23 year old strain cultivated for 5 days in 33 per cent embryo juice. $\times$ 115.

FIG. 5. The same cells as shown in Fig. 4 after 16 days cultivation in 33 per cent embryo juice. $\times$ 115.

FIG. 6. Cells from a sister colony to that shown in Fig. 5 cultivated for 16 days in a mixture of 33 per cent embryo juice and 8 per cent serum. Cells near the center of the colony. $\times$ 115.

FIG. 7. Cells at the periphery of the colony shown in Fig. 6, after 16 days cultivation in 33 per cent embryo juice and 8 per cent serum. Such a network of cells covered the entire flask. $\times$ 115.

PLATE 37
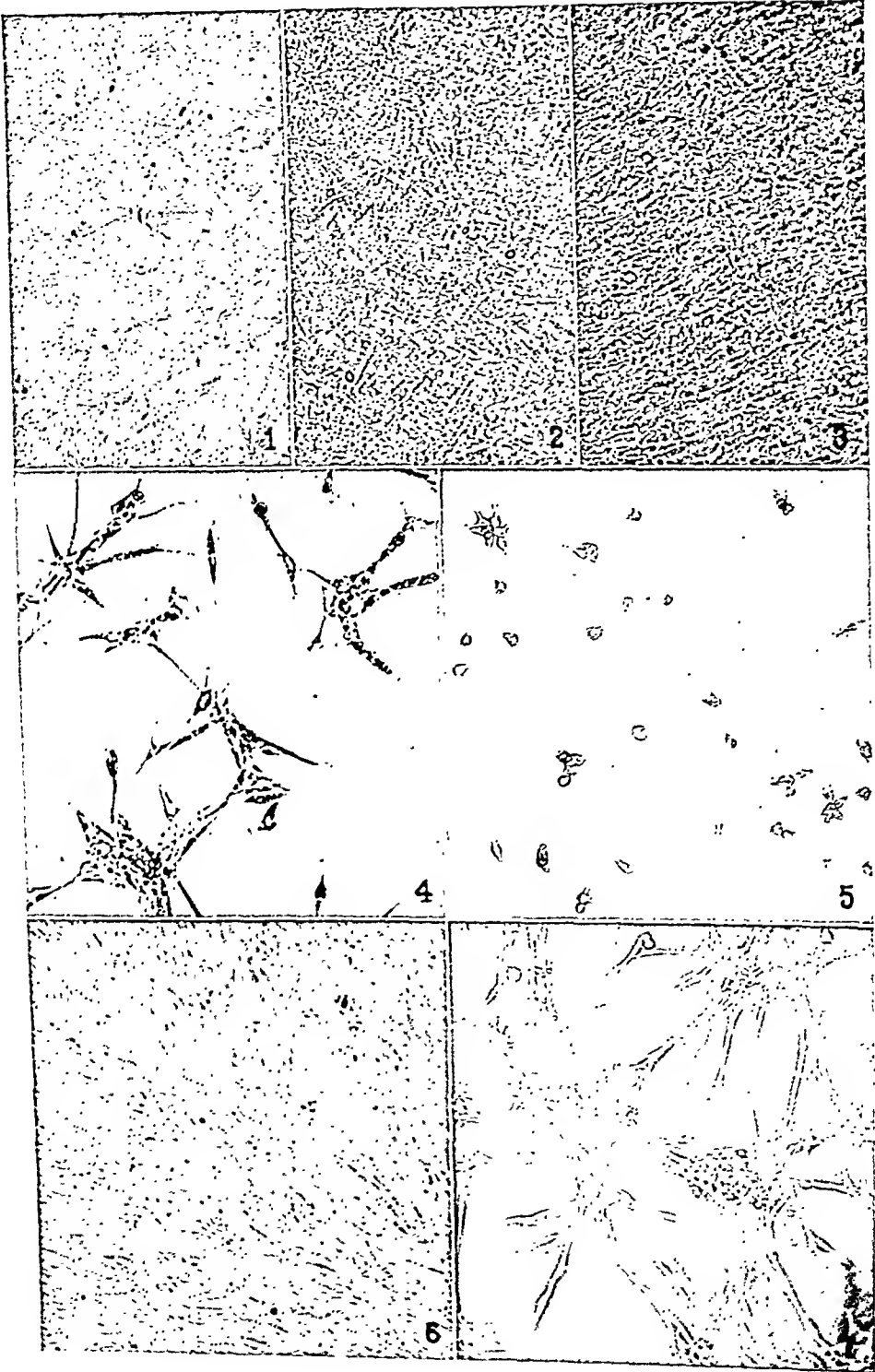
THE ENHANCING EFFECT OF AZOPROTEINS ON THE LESIONS PRODUCED BY VACCINE VIRUS, THE SHOPE FIBROMA VIRUS, AND THE AGENT TRANSMITTING CHICKEN TUMOR I

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It has been noted that the lesions produced in the skin of rabbits by the injection of various infectious agents are markedly enhanced by the addition of testicular extract (1). The phenomenon is now known to depend on a factor which causes intradermally injected material to spread extensively through the dermis (2). A similar factor capable of increasing tissue permeability can be detected in extracts derived from a variety of other animal tissues and from certain microorganisms (3). Azoproteins have been shown to produce a like effect, as far as increasing skin permeability is concerned (4), but whether or not the mechanism involved in the production of the spread is the same for the two classes of substances, has not been determined (5). For further comparison the effect of azoprotein solutions has been tested on the lesion produced by vaccine virus, the Shope fibroma virus, and the agent transmitting Chicken Tumor I.

Material and Method

Azoproteins.—The azoproteins were prepared by following closely the procedure given in a previous paper (4). In the present work the azo compounds used were produced by the coupling of *p*-diazobenzenesulfonic acid with horse or rabbit serum proteins, or with egg albumin. Gelatin, which is practically lacking in coupling power, was also used in a few tests. Neutralized solutions of *p*-diazobenzenesulfonic acid, serum, and egg albumin solutions at the proper concentrations, were tested separately as controls.

Test for Spreading Power.—As a rule, the spreading power of the azoprotein solutions tested for enhancing properties was also determined, usually in the same animal, and in a corresponding area of the skin. For these tests, 0.5 cc.

of the azoprotein solution was mixed in a syringe with 0.25 cc. of India ink previously diluted with 2 volumes of water, and the mixture was injected intracutaneously. The results were recorded by measuring the area of spread 18 to 24 hours after the injection. Since azoproteins are colored products, their spread through the dermis could be estimated also directly, through discoloration of the skin in the area of diffusion, without need for the addition of India ink.

Vaccine Virus.—The vaccine virus used in the present tests was a subculture of an *in vitro* strain (B. P. H. culture 662, Oct. 16, 1934), kindly supplied by Dr. T. M. Rivers. The infective material had been stored in 50 per cent glycerin and, before use, was diluted with Ringer's solution.

Shope Papilloma Virus.—The original fibroma virus was kindly supplied by Dr. R. Shope (6). The material used in these experiments was obtained by the subpassage of the virus in the skin of rabbits. The freshly removed fibroma tissue was ground with sand and extracted with distilled water in the proportion of 5 to 10 cc. water per each gram of tissue. The extract was then centrifuged and filtered through sterile gauze.

Chicken Tumor Agent.—Fresh Chicken Tumor I tissue was ground with sand and extracted with sterile distilled water in the proportion of 10 to 12 cc. water per gram of tissue. After centrifugation at 3000 R.P.M. for 10 minutes, the extract was filtered through sterile gauze. In some instances the inhibiting factor was removed from the extract by adsorption with aluminum hydroxide (7).

Tests for Enhancing Power of Azoprotein Solutions.—The enhancing property was tested by mixing an equal volume of the azoprotein solution with dilutions of the virus suspensions. Of these mixtures 1 cc. was injected subcutaneously in rabbits. Virus preparations diluted with an equal volume of saline solution or with untreated protein solutions served as controls.

The chicken tumor extract was mixed in the syringe with an equal volume of the test solutions, and 0.6 to 0.8 cc. of the mixture was injected intracutaneously into adult Plymouth Rock hens. Since tissue diffusion caused both by testicular extract and azoproteins is influenced by gravity, in order to allow sufficient space for the material to spread, the injections were made just under the wing or in the skin covering the upper part of the leg.

Effect of Azoproteins on Vaccine Virus Lesions

The results of tests with azoproteins and varying dilutions of vaccine virus compared with the areas of spread of the azoprotein with India ink are shown in Table I.

It will be noted that azoprotein increases to a considerable degree the size of the lesion produced by vaccine virus. The results indicate that the extent of the lesion depends not only on the spreading power of the azoprotein, but also on the potency of the infective material. As a rule, the more concentrated virus preparation with azoprotein gave, 6 to 7 days after inoculation, wide, solid lesions surrounded

by a zone of edema. This condition is illustrated in Fig. 1, where the size of the enhanced lesions was 67.6 sq. cm. compared with 10.5

TABLE I

Effect of Azoproteins on the Size of the Lesions Produced by Vaccine Virus in the Rabbit Skin

Experiment No.	Azoproteins prepared from sulfanilic acid and	Dilution of vaccine virus	Area of spread of India ink indicator and		Area of 7 day old lesions produced by vaccine virus and		Character of virus lesions on azoprotein side
			Saline	Azoprotein solution	Saline	Azoprotein solution	
			sq.cm.	sq.cm.	sq.cm.	sq.cm.	
1	Egg albumin (3% sol)	1/10	5.5	87.0	10.5	67.6	Solid mass surrounded by scattered nodules and zone of edema
2	" " " "	1/10	4.2	60.7	14.0	34.0	Solid mass
	" " " "	1/100			7.5	27.6	Only scattered nodules
	" " " "	1/1000			0	16.6	" " "
3	Gelatin (3% sol)	1/10	6.8	41.8	12.4	0	No lesions (virus inactivated)
4	Horse serum	1/10	1.8	90.3	6.7	82.8	Solid mass surrounded by scattered nodules and edema
5	" "	1/10	8.0	76.7	4.6	44.6	Solid mass
	" "	1/100			2.2	25.5	Solid mass plus scattered nodules
	" "	1/1000			0	17.0	Scattered nodules
6	" "	1/10	6.2	58.0	20.8	56.0	Solid mass
	" "	1/100			10.2	46.8	Solid mass plus scattered nodules
	" "	1/1000			1.8	48.1	Only scattered nodules
7	Rabbit serum	1/10	3.4	36.8	6.7	39.1	Solid mass
	" "	1/100			3.4	27.6	Solid mass plus scattered nodules
	" "	1/1000			2.1	20.8	" "

sq. cm. for the saline control. The azoprotein used with the vaccine virus was tested for spreading power in another rabbit and gave, 24

hours after the injection, an area of spread of 87 sq. cm. against 5.5 sq. cm. for the saline control (Fig. 2).

The injection of dilute virus with azoprotein gave rise to scattered nodules, with or without a solid lesion at the site of injection.

In two instances, vaccine lesions developed from the azoprotein-virus mixtures while the virus-saline inoculum remained negative. This evidence of real enhancement of the virus, as the result of the spread, was also observed with testicular extract by Duran-Rey-

TABLE II

Effect of Azoproteins on the Size of Fibroma (Shope) in the Rabbit Skin

Experiment No.	Azoprotein prepared from sulfanilic acid and	Area of spread of India ink indicator plus		Area of 7 day old lesions produced by fibroma virus plus		Character of lesions on azoprotein side
		Saline or normal serum	Azo-protein solution	Saline or normal serum	Azo-protein solution	
		sq.cm.	*sq.cm.*	*sq.cm.*	*sq.cm.*	
1	Horse serum	7.5	77.0	5.2	71.4	Solid mass, surrounded by a few scattered nodules
2	" "	5.7	43.1	4.0	35.7	Solid tumor
3	" "	6.1	62.4	5.6	54.3	Solid tumor, surrounded by isolated nodules

nals (8).   It is possible that the presence of the tissue extract or the azoprotein solution stabilizes the virus at the highest dilutions, and protects it from inactivation.   Such an action would explain the apparent enhancement of the virus itself.

Azoproteins prepared from serum and from egg albumin were identical in their effect.   A gelatin solution, submitted to the same procedure of coupling exhibited some spreading power, but the vaccine virus when added to the latter, was completely inactivated. The virus was also destroyed by the neutralized solution of the diazo compound.   These findings are in agreement with the earlier observations that diazo compounds or the product of coupling with gelatin are antiseptic (4).

Effect of Azoproteins on Rabbit Fibroma (Shope)

The results given in Table II were recorded 6 to 7 days after the injection of the infective material into the rabbit skin.

It will be seen from the table that injections of 1 cc. of the fibroma extract diluted with saline gave rise to small tumors, 2 to 2.5 cm. in diameter. The same amount of virus to which azoprotein solution had been added, produced extensive tumor formation. These were large masses of solid growth surrounded by a few scattered nodules. A typical example of this result is shown in Fig. 3. The injection of azoprotein and India ink in the same animal (Fig. 4) shows that the area of spread and the size of the tumor are practically the same.

TABLE III

Effect of Azoproteins on the Size of Skin Tumors in Chickens

Experiment No.	Azoprotein prepared from sulfanilic acid and	Apparent area of spread		Enhancement of tumors			Aspect of tumor on azoprotein side
					Average size of tumors		
		Saline (control)	Azoprotein solution	Age of tumors	Saline (control)	Azoprotein solution	
		sq.cm.	*sq.cm.*	*days*	*sq.cm.*	*sq.cm.*	
1	Egg albumin (3% sol)	3.2	25.5	16	7.3	44.8	Solid tumor surrounded by scattered nodules
2	Horse serum	3.7	19.2	21	4.0	16.4	Massive tumor
	" "	4.1	55.1	21	3.9	49.6	Solid mass plus small nodules scattered around main tumor
3	" "	3.5	39.9	16	5.8	36.8	Solid tumor
4	" "	2.5	36.0	17	6.4	57.8	" "
5	" "			15	6.0	24.0	Scattered nodules only
	" "			15	6.1	30.4	" " "

Effect of Azoproteins on Chicken Tumors

The effect of testicular extract on the development of skin tumors in chickens has been described in a previous paper (9). Table III shows the results obtained when azoprotein solutions were added to the tumor material before injection. As in the preceding cases, azoprotein solutions caused the tumor agent to spread over a large area of the skin, giving rise to large, massive tumors when the tumor

extract was very active, and to widely scattered nodules when the extract had a low tumor producing activity. The diffusion of the azoprotein solution in the chicken skin could be observed without the addition of India ink as an indicator. There was found to be a good agreement between the extent of the spread and the size of the tumors. Figs. 5 and 6 show tumors produced by a highly active extract and azoproteins, 16 days after the injection. Fig. 7 shows the effect of dispersion on material of low tumor producing activity.[1]

SUMMARY

It is known that azoprotein solutions, like testicular extracts, possess the property of causing particles to spread through the dermis. The present work shows that azoproteins exhibit, like testicular extract, the power to increase the size of virus lesions in the skin of rabbits, and the size of tumors in chickens. The results indicate that the extent of the lesion is roughly proportional to the spreading power of the solution. This suggests that the spread of the infective material, over a larger area of skin, is directly responsible for the enhancing effect.

The production of extensive lesions by means of spreading agents may have a practical value when large amounts of working material are needed.

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[1] In addition to the above tests, the effect of azoproteins was tried on mouse and rabbit tumors. Duran-Reynals has shown that testicular extract mixed with the tumor cells would inhibit the development of Brown-Pearce tumors in the skin of rabbits (10). Azoproteins had no such effects on the growth of mouse tumor 48 or on the Brown-Pearce tumor. On the contrary a slight enhancing effect on the growth of these tumors was observed by Dr. Van der Schueren in this laboratory. However, it was noted that, when an azoprotein solution was mixed with the tumor cells, the material turned into a soft gel, and it is possible that this condition interfered with the usual spreading property of the compound.

3. Claude, A., and Duran-Reynals, F., *J. Exp. Med.*, 1934, **60**, 457. Duran-Reynals, F., *J. Exp. Med.*, 1933, **58**, 161.
4. Claude, A., *J. Exp. Med.*, 1935, **62**, 229.
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6. Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793.
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10. Duran-Reynals, F., *J. Exp. Med.*, 1931, **54**, 493.

EXPLANATION OF PLATES

PLATE 38

FIG. 1. Rabbit 9-50. Lesions produced by the intradermal injection of 0.5 cc. vaccine virus suspension mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from egg albumin.

FIG. 2. Rabbit 9-49. Spread produced by intradermal injection of 0.5 cc. India ink suspension mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from egg albumin. The area of spread. 24 hours after injection, was 87.0 sq. cm. for the azoprotein solution and 5.5 sq. cm. for the saline control.

FIG. 3. Rabbit 9-21 (right side). Skin fibroma produced by the intradermal injection of 0.5 cc. suspension of Shope fibroma virus mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from horse serum.

FIG. 4. Rabbit 9-21 (left side). Spread produced by intradermal injection of 0.5 cc. of an India ink suspension mixed with an equal volume of: A, saline solution (control) and B, azoprotein solution prepared from horse serum. The area of spread 24 hours after injection was 7.5 sq. cm. for the control and 77.0 sq. cm. for the azoprotein solution.

PLATE 38

(Claude. Enhancing effect of azoproteins on lesions)

PLATE 39

FIG. 5. Chicken S-84. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed, in the syringe, with an equal volume of: A, saline solution (control) and B, azoprotein solution prepared from horse serum. The size of the tumor, recorded 16 days after injection, was 2.2 × 2.1 cm. for the saline control and 7.7 × 6.8 cm. for the azoprotein mixture.

FIG. 6. Chicken S-73. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed with: A, saline solution (control) and B, azo-protein solution prepared from horse serum. 21 days after injection there was a main tumor, 6.5 × 6.5 cm. at the site of injection, surrounded by smaller nodules scattered over an area 9.2 × 6.7 cm. The tumor produced by the saline mixture measured 2.2 × 2.1 cm.

FIG. 7. Chicken S-69. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from horse serum. In this case, the tumor extract was not very active and the lesions produced by the azoprotein mixture were represented by small isolated tumors scattered over an area 9.0 × 4.7 cm. The control mixture produced a solid tumor, 2.8 × 2.7 cm. across.

PLATE 39
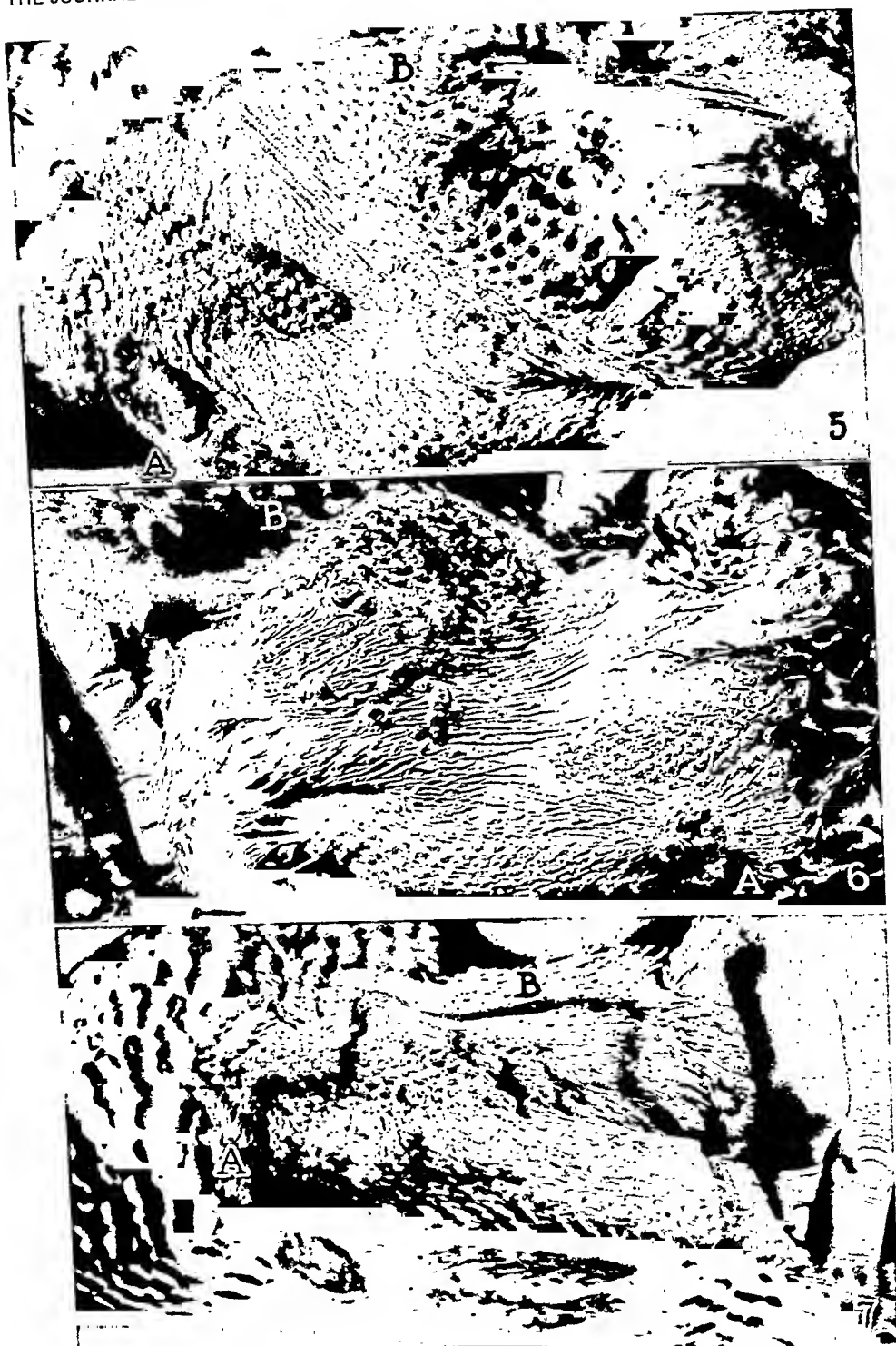
(Claude: Enhancing effect of azoproteins on lesions)

STUDIES ON EXPERIMENTAL HYPERTENSION

IX. The Effect on Blood Pressure of Constriction of the Abdominal Aorta above and below the Site of Origin of Both Main Renal Arteries[*][†]

By HARRY GOLDBLATT, M.D., JOSEPH R. KAHN, M.D., and RAMON F. HANZAL, Ph.D.

(From the Institute of Pathology, Western Reserve University, Cleveland)

A method has been described for the production of hypertension in dogs by the constriction of the main renal artery of one or both kidneys (2, 3). When the main renal artery of only one kidney is constricted, the blood pressure rises, but after a variable period returns to the original level. When both main renal arteries are adequately constricted, or if one is constricted and the other kidney is removed, the blood pressure usually remains elevated. This has been accomplished successfully in dogs (3) and in monkeys (4), and the method, or some modification of it, has now been used by ourselves (2–11) and others (12–93) in the production of hypertension by renal ischemia and in the investigation of the pathogenesis and treatment of this type of hypertension, as well as the application of the results to the problem of human hypertension.

In small laboratory animals, like monkeys, cats, rabbits and rats, it is rather difficult to constrict adequately without frequently occluding the main renal artery. It occurred to us that if moderate constriction of the aorta just above the origin of both main renal arteries would result in hypertension, at least above the site of the clamp, and if constriction immediately below the origin of the renal arteries would have no effect on the blood pressure, this would indicate that

the mechanical obstruction of the aorta is not the cause of such hypertension. Moreover, if renal excretory insufficiency, as well as hypertension, would result from very great constriction of the aorta above the origin of both main renal arteries, as it does from very great constriction of the main renal arteries (3, 7, 9), this type of hypertension, with or without renal insufficiency, could also be considered of renal origin. This method could then be used instead of constriction of the main renal arteries for the production of hypertension of renal origin, at least in the upper part of the body of small animals. Incidentally, this method might afford an opportunity to study in the same animal the possible effects on aorta, arteries and
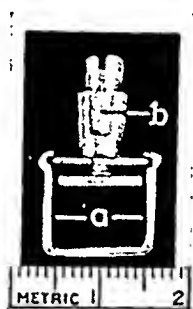
TEXT-FIG. 1. Clamp for aorta, inside diameters 1 cm.

(*a*) Right-angled U-shaped portion, the receptacle for the artery.

(*b*) Removable portion, consisting of screw stem carrying two plates. The upper plate slides into grooves in U-shaped part to enclose aorta. The lower plate can be screwed down to constrict the vessel.

arterioles of hypertension above and hypotension below the level of constriction of the aorta. Also, by applying the clamp to the aorta at a higher level, even within the thorax, some information might be obtained about the pathogenesis of hypertension usually found above the site of coarctation or stenosis, especially in the thoracic portion of the aorta in man.

Experimental Methods

Animals.- Dogs were used. They were of similar types, kept under the same conditions and fed the same diet as in previous experiments (2, 11).

The Clamp. The clamp used for the constriction of the dog's aorta is larger and of somewhat different construction from the renal clamp (3). It is made of silver or stainless steel and consists of a right-angled U-shaped portion, the

open receptacle for the artery (Text-fig. 1 *a*), and a removable portion consisting of a screw stem carrying two plates (Text-fig. 1 *b*). The upper plate slides into a groove on the inner sides of the U-shaped part and serves to enclose the aorta. The lower, movable plate is attached to a double-acting screw and can be screwed down to constrict the aorta to any desired degree. A special instrument devised for the purpose of holding the U-shaped part facilitates the application of this part of the clamp on the aorta and also aids the insertion of the screw stem with the double plate into the U-shaped portion, to enclose the aorta within the clamp. This instrument is not necessary, but it simplifies the procedure.

Surgical Procedure for the Constriction of the Aorta.—All the operations but one were performed under ether anesthesia, with a previous hypodermic injection of morphine and atropine. On one dog (No. 4-13), the operation was performed under local anesthesia with procaine, after a hypodermic injection of morphine and atropine.

For the constriction of the aorta above the origin of both main renal arteries, an oblique incision about 6 cm. in length is made in the skin about 1.5 cm. below and parallel to the right inferior costal margin, beginning at the costolumbar angle. This approach is the same as is used for the application of a clamp on the right main renal artery (3), or for right nephrectomy or suprarenalectomy (7). A gridiron incision in the internal oblique and transversalis muscles, lateral and posterior to the right border of the external oblique muscle, reveals peritoneum, which is then pushed away manually from its loose attachment to the underlying tissues of the lateral wall of the abdomen. A self-retaining retractor with long thick wire blades is then inserted to retract the wound and keep it open. The peritoneal covering of the aorta is incised on the right side, just above the origin of the right main renal artery, and the aorta immediately above the origin of this vessel is then exposed and dissected away for a distance of about 1 cm. from its posterior attachment to the tissues in front of the spine. In some cases, when they are situated close to the right main renal artery, it is necessary to ligate and sever two lumbar arteries which originate from the aorta posteriorly between the levels of the superior mesenteric and right main renal artery. This must be done in order to make room for the clamp which is applied to the aorta just proximal to the origin of the right main renal artery.

As soon as the aorta is enclosed in the clamp, a 20 gauge needle connected with a mercury manometer is inserted in the femoral artery to measure direct or so called mean blood pressure. The degree of constriction of the aorta is determined by the immediate effect on the femoral mean pressure and it can be varied at will in different animals. In these experiments the constriction was usually made adequate to reduce the femoral mean pressure to 50 per cent, or less, of the normal for the animal. Closure of the incision is effected by means of continuous catgut sutures for the muscle and subcutaneous tissue and continuous black silk sutures for the skin.

For the application of a clamp on the aorta just below both main renal arteries, the incision is made on the left side, and the clamp is placed just below the site of

origin of the left main renal artery.   This portion of the aorta is free of any other arterial branches, so that it is not necessary to sever any vessels to make room for the clamp.   Otherwise the operation is similar to that described for the application of the clamp above the origin of both main renal arteries.   The incision on the left side is made a little lower than on the right (about 2 cm. below and parallel to the costal margin).   It is sometimes necessary also to split the external oblique muscle in making the gridiron incision on the left side.

Measurement of Blood Pressure.—In all the animals blood pressure was determined by two methods.   Carotid systolic pressure was measured by the van Leersum carotid loop method (94), and direct or mean pressure was measured by inserting into the carotid or femoral artery a needle connected by means of a liquid system (2 per cent sodium citrate) with a mercury manometer.   During the operation for constriction of the aorta a 20 gauge needle was used, but for other determinations of femoral mean pressure, the needle was 21 gauge.   Normal blood pressure was determined frequently by both methods for at least 1 month before the aorta was constricted.

The Effect of Constriction of the Aorta Just above the Site of Origin of Both Main Renal Arteries

Constriction of the aorta just above the origin of both main renal arteries, or above the functioning main renal artery in a unilaterally nephrectomized animal, invariably resulted in lowering of the femoral mean pressure and elevation of carotid systolic or carotid mean pressure.   The effect on the blood pressure in the carotid loop was not immediate, but developed in about 24 to 48 hours, that is, in about the same time as following constriction of one or both main renal arteries (3).   The reduction of femoral mean pressure was immediate and varied with the degree of constriction of the aorta.   In many instances the femoral mean pressure rose later, coincidentally with the rise of pressure in the carotid loop, and in about 1 or 2 weeks reached its maximum level, usually the previous normal.   In some instances, especially if the aorta below the origin of both renal arteries was also constricted, even the femoral mean pressure finally reached levels higher than the normal.

A. The Development of Hypertension without Renal Excretory Insufficiency (the Benign Phase)

In four animals (Nos. 3-21, 3-49, 3-51 and 3-60) the carotid systolic pressure became greatly elevated as a result of constriction of the aorta just above the origin of both main renal arteries, and the

hypertension persisted until death, without accompanying renal excretory insufficiency.   Three of these animals died of intra-abdominal hemorrhage due to erosion of the wall of the aorta by the clamp.   The longest survival was 25 days.   In one animal, (No. 3-60), that survived 8 days with elevated blood pressure, after constriction of the aorta above the origin of both renal arteries, the cause of death was not determined at autopsy.

To save space, the experimental history of only the most important animals will be given in full detail, the others will be abbreviated.

No. 3-21: Mongrel, short haired, female, young, 14.2 kg.

Mar. 16 to July 28, 1937. During this pre-operative period the systolic blood pressure in the carotid loop (carotid systolic pressure) varied between 140 and 160 mm. Hg, and the mean pressure in the femoral artery (femoral mean pressure) varied between 120 and 140 mm. Hg.

July 28. The aorta was moderately constricted by a clamp applied just above the origin of both main renal arteries. *July 29 to Aug. 5.* During this period the carotid systolic pressure varied between 220 and 280 mm. Hg.   On July 29, 1937, 24 hours after the constriction of the aorta, the carotid systolic pressure was 276 mm. Hg.   The femoral mean pressure, which was 80 mm. Hg immediately after the constriction of the aorta, rose to 90 mm. Hg on Aug. 3. *Aug. 6.* At 9 a.m. the carotid systolic pressure had dropped to 135 mm. Hg. The animal appeared ill.   Blood urea nitrogen (B.U.N.) 20.3 mg., non-protein nitrogen (N.P.N.) 35.4 mg., creatinine (Cr.) 1.3 mg. and CO_2 combining power (CO_2) 24.2 volumes per 100 cc. of plasma.   At 3 p.m. the animal was found dead. At autopsy, the cause of death was found to have been erosion of a lumbar artery by one edge of the clamp which impinged on this vessel.

No. 3-49: Hound, female, old, 14.0 kg.

July 6 to Sept. 16, 1937. During this pre-operative period the carotid systolic pressure varied between 134 and 152 mm. Hg, and the femoral mean pressure varied between 128 and 144 mm. Hg. *Sept. 16.* The aorta was greatly constricted by a clamp applied just above the origin of both main renal arteries. *Sept. 17 to Oct. 10.* During this period the carotid systolic pressure varied between 240 and 284 mm. Hg.   On Oct. 9, 2 days before death, it was still 260 mm. Hg. The femoral mean pressure was 75 mm. Hg immediately after constriction of the aorta and rose to a maximum of 130 mm. Hg on Sept. 27. *Oct. 11.* The animal was found dead.   At autopsy, the cause of death was found to have been due to erosion of the aorta by the clamp.

No. 3-51: German shepherd, male, middle age, 22.0 kg.

July 9 to Sept. 21, 1937. During this pre-operative period the carotid systolic

pressure varied between 144 and 166 mm. Hg, and the femoral mean pressure varied between 115 and 135 mm. Hg.

Sept. 21. The aorta was greatly constricted by a clamp applied just above the origin of both main renal arteries. *Sept. 22 to Sept. 30.* During this period the carotid systolic pressure varied from 210 to 260 mm. Hg. On Sept. 30, it was still 230 mm. Hg. *Oct. 1.* The animal was found dead. At autopsy, the cause of death was found to have been intra-abdominal hemorrhage caused by erosion of the aorta by the clamp.

No. 3-60: Black and white short haired mongrel, female, young, 11.8 kg.

In this animal, the carotid systolic pressure rose from the normal of 150 to 170 mm. Hg (Sept. 16 to Oct. 27, 1937) to a maximum of 256 mm. Hg, on Nov. 3, 1 week after the constriction of the aorta above the origin of both main renal arteries. Femoral mean pressure fell from the normal of 130 to 65 mm. Hg, immediately after the clamp was applied, but rose to 105 mm. Hg on Nov. 3. The animal was found dead on Nov. 5. 2 days after the operation there was no chemical evidence of renal excretory insufficiency, but during the last 7 days of life chemical examination of the blood was not made. The cause of death was not determined. There were no lesions suggestive of renal insufficiency complicating the hypertension.

B. The Development of Hypertension, Renal Excretory Insufficiency, and Fatal Convulsive Uremia with Degenerative, Necrotizing and Inflammatory Arteriolar Disease in Many Organs (the Acute Malignant Phase)

Three animals (Nos. 3-37, 3-59, 3-63) with the aorta greatly constricted just above the origin of both main renal arteries, died in uremia, a variable period after application of the clamp. The uremia was obviously due to renal excretory insufficiency caused by marked renal ischemia, the result of sudden excessive constriction or actual occlusion of the aorta. In all the animals the carotid systolic pressure rose progressively to high levels as the degree of uremia also increased. At autopsy, in the gross, some of the uremic animals had petechiae in various organs and, microscopically, showed arteriolar degeneration and necrosis, as well as acute arteriolitis in these organs, principally the alimentary tract (except rectum), gall bladder, pancreas, myocardium and brain. This was similar to the effect of excessive constriction or occlusion of the main renal arteries (the acute malignant phase) previously described (9).

No. 3-37: Collie, female, young, 14.4 kg.

Apr. 20 to Oct. 27, 1937. During this pre-operative period the carotid systolic

pressure varied between 132 and 158 mm. Hg, and the femoral mean pressure varied between 112 and 125 mm. Hg. On Oct. 27, B.U.N. 15.7 mg., Cr. 1.3 mg., CO_2 49.4 volumes per 100 cc. of plasma.

Oct. 27. The aorta was greatly constricted by a clamp applied just above the origin of both main renal arteries. The femoral mean pressure was reduced immediately from 115 to 55 mm. Hg, and it remained at this level until death. During the 3 days after the constriction of the aorta, the carotid systolic pressure varied between 198 mm. Hg, 24 hours after the operation, and 245 mm. Hg on Oct. 29, the day before death. During this period the animal became progressively more uremic. *Oct. 29.* B.U.N. 128 mg., Cr. 7.6 mg., CO_2 28.6 volumes per 100 cc. of plasma.

Oct. 30. The animal was found dead. At autopsy, in the gross, there were petechiae in the stomach, small and large intestine and pancreas. Microscopically, these petechiae were associated with arteriolar degeneration and necrosis, but many were obviously of capillary origin.

No. 3-59: Mongrel collie, female, 10.4 kg.

Sept. 7 to Oct. 20, 1937. During this pre-operative period the carotid systolic pressure varied between 143 and 156 mm. Hg, and the femoral mean pressure varied between 115 and 135 mm. Hg.

Oct. 20. The aorta was greatly constricted by a clamp applied just above the origin of the main renal arteries. The femoral mean pressure was reduced immediately from 115 to 50 mm. Hg. *Oct. 21 to Oct. 24.* During this time the carotid systolic pressure rose progressively from 188 mm. Hg, 24 hours after the operation, to 282 mm. Hg on Oct. 23. On Oct. 24, B.U.N. 104 mg., Cr. 6.8 mg.

Oct. 25. The animal was found dead. At autopsy, in the gross, there were petechiae, some confluent, in the mucosa and serosa of stomach, small and large intestines and gall bladder, and also in the pancreas and epicardium. The hemolymph nodes were intensely hyperemic. Microscopically, in the organs that showed gross petechiae and in some that did not, many arterioles were the seat of hyalinization, fibrinoid degeneration or necrosis. In many instances the extravasation of blood appeared to be of capillary origin.

No. 3-63: Chow, female, middle age, 19.6 kg.

In this animal also the carotid systolic pressure rose from the normal of 151 to 168 mm. Hg (Sept. 22 to Dec. 22, 1937), to 272 mm. Hg on Dec. 24, 2 days after the constriction of the aorta. Femoral mean pressure fell from the normal of 130 to 155 mm. Hg, to 75 mm. Hg, immediately after the constriction of the aorta, and it remained at this level. The nitrogenous products of the blood rose, and CO_2 combining power fell, progressively, until on Dec. 26, they were, B.U.N. 188 mg., Cr. 6.0 mg. and CO_2 30.3 volumes per 100 cc. of plasma. On Dec. 27, the animal was found dead. In the gross, and microscopically, the typical lesions of the malignant phase were found in the small and large intestine and pancreas. In the liver, hyalinized arterioles without accompanying petechiae were found.

The Effect of Constriction of the Aorta above the Origin of Both Main Renal Arteries Followed by Constriction of the Aorta below the Renal Arteries and Finally Constriction of the Main Renal Arteries

In those animals that survived several weeks or months the constriction of the abdominal aorta above the origin of both main renal arteries, there was a tendency for the elevated carotid systolic or carotid mean pressure to return to a lower or even to the normal level. Increased constriction, and finally occlusion, of this part of the aorta was followed by re-elevation of the carotid systolic or carotid mean pressure, which, after a variable time, again tended to fall. In such an animal (No. 3-50), subsequent constriction, and then occlusion of the aorta just below the origin of both renal arteries caused a temporary re-elevation of the carotid systolic pressure. Even femoral mean pressure reached a level above normal. Constriction of one main renal artery of such an animal again re-elevated the pressure, but it was necessary to constrict, or even gradually to occlude, both main renal arteries, in order to effect marked renal ischemia and persistent elevation of carotid and femoral blood pressure at a high level.

No. 3-50: Collie, mongrel, female, middle age, 14.1 kg.

July 16 to Sept. 14, 1937. During this pre-operative period the carotid systolic pressure varied between 134 and 165 mm. Hg, and the femoral mean pressure varied between 110 and 135 mm. Hg.

Sept. 14. The aorta was moderately constricted just above the site of origin of both main renal arteries. The femoral mean pressure fell immediately from 125 mm. Hg to 90 mm. Hg. *Sept. 15.* Carotid systolic pressure rose to 204 mm. Hg. *Sept. 16 to Oct. 18.* Carotid systolic pressure rose to a maximum of 246 mm. Hg, on Sept. 27, and then gradually fell to 187 mm. Hg, on Oct. 18. The femoral mean pressure gradually rose to 145 mm. Hg.

Oct. 18, 1937, to Nov. 22, 1938. During this period, the aorta above both main renal arteries was occluded, on Oct. 18, and the aorta below both main renal arteries was first constricted, on Dec. 15, 1937, and then occluded, on Mar. 3, 1938. During the period after each operation, the carotid systolic pressure rose and then gradually fell again.

Then in succession the renal arteries were first constricted and finally occluded. The left main renal artery was constricted, June 9, 1938, and occluded Oct. 27. The right main renal artery was constricted on June 30, and occluded on Nov. 22.

Nov. 23 to Dec. 24. The carotid systolic pressure rose to 304 mm. Hg on Nov. 25, and it has remained greatly elevated. Even femoral mean pressure rose to 200 mm. Hg on Nov. 11, despite the occlusion of the aorta above and

below the main renal arteries. Chemical examination of the blood showed
B.U.N. 15.7 mg., Cr. 1.3 mg., CO_2 47.0 volumes per 100 cc. of plasma.   The urea
clearance was 24.1 which was about 60 per cent of the normal for this dog, and
indicated moderate impairment of renal excretory function, although it was not
obvious from the determinations of urea and creatinine in the blood.   On Dec. 24,
femoral mean pressure was still 190 mm. Hg, while the carotid systolic pressure
was 285 mm. Hg.   On this date, B.U.N. 18.8 mg., Cr. 1.7 mg., CO_2 48.3 vol-
umes per 100 cc. of plasma.   In a concentration test the specific gravity of the
urine was 1.025.   The animal is still living.

The Effect of Releasing the Clamp on the Aorta Constricted above the Origin of the Main Renal Arteries

In one animal (No. 3-71), the release of the clamp on the aorta just
above the origin of both main renal arteries was followed within 24
hours by a fall of the elevated carotid systolic pressure to the original
level.   The femoral mean pressure returned to normal immediately
after the release of the constriction.

No. 3-71: Doberman pinscher, male, old, 22.8 kg.
Oct. 30 to Dec. 28, 1937.   During the pre-operative period the carotid systolic
pressure varied between 154 and 172 mm. Hg.   The femoral mean pressure
varied between 140 and 155 mm. Hg.
Dec. 28.   The aorta was greatly constricted just above the origin of both
main renal arteries.   The femoral mean pressure was reduced immediately from
140 to 70 mm. Hg.
Dec. 29.   24 hours after the operation the carotid systolic pressure was 248
mm. Hg.   The dog's hind limbs were paralyzed, the anus was incontinent, and
some bloody fluid material kept passing out from the intestine.   Under local
anesthesia, the aortic clamp was removed.   The carotid systolic pressure re-
mained elevated at 240 mm. Hg immediately after removal of the clamp.   *Dec. 30,*
1937, to Jan. 9, 1938.   24 hours after the operation the carotid systolic pressure
was 156 mm. Hg, which was well within the normal range for this animal, and
it remained down for the next 9 days.   2 days before death the carotid systolic
pressure was 148 mm. Hg, and the femoral mean pressure was 130 mm. Hg.
Jan. 10.   The animal was found dead.   The cause of death was not determined.
There were no lesions suggestive of uremia.

The Effect of Constriction of the Abdominal Aorta Just below the Origin of Both Renal Arteries Followed Later by Constriction of the Aorta above the Origin of Both Main Renal Arteries

In order to constrict the aorta just below the origin of both main
renal arteries, it was necessary to apply the clamp just below the

origin of the left main renal artery. Full details about the method of application of the clamp have already been given.

Constriction of the aorta just below the origin of both main renal arteries had no significant effect on the carotid systolic blood pressure of animals with two kidneys or of unilaterally nephrectomized animals. When, however, constriction of the aorta below the origin of the main renal arteries was followed by constriction of the abdominal aorta just above the origin of both main renal arteries, a significant elevation of blood pressure always occurred. The hypertension was usually higher and lasted longer than when the aorta was constricted only above the site of origin of the main renal arteries. However, even the constriction of the aorta below and above the main renal arteries was followed, after a variable period, by a lowering of the elevated blood pressure, or a return to the original level. Increased constriction or occlusion of the abdominal aorta above and below the origin of the main renal arteries caused significant but temporary re-elevation of the blood pressure. In some of these animals, even the femoral mean pressure reached a level significantly higher than the normal. Three of these animals (Nos. 3-44, 3-91, 3-92) finally died in uremia due to renal excretory insufficiency caused by sudden, excessive constriction of the aorta above the origin of the main renal arteries. These animals developed the necrotizing and inflammatory arteriolar disease previously described (9) in many of the organs in which the arterial pressure was elevated. This result was similar to that of some of the animals with the aorta greatly constricted only above the origin of the main renal arteries.

One animal (No. 3-57) with the aorta constricted first below and then above the main renal arteries, died of intra-abdominal hemorrhage, due to erosion of the aorta above the origin of both main renal arteries. This animal lived 9 weeks after the constriction of the aorta below the main renal arteries, and 19 days following the constriction of the aorta above the main renal arteries. During the latter period the carotid systolic pressure remained elevated considerably above the normal. Even the femoral mean pressure finally became elevated above the original level.

In a unilaterally nephrectomized animal (No. 3-89) the application of the clamp on the aorta below the main renal artery of the remain-

ing kidney was not followed by elevation of the carotid systolic pressure.

No. 3-44: White spitz, female, young, 14.0 kg.

June 22 to Sept. 13, 1937. During this pre-operative period the carotid systolic pressure reached 172 mm. Hg, but during the last 2 weeks of this period, it varied between 141 and 152 mm. Hg. The femoral mean pressure varied between 115 and 145 mm. Hg.

On *Sept. 13*, the aorta was greatly constricted just below the origin of both main renal arteries. The femoral mean pressure was reduced immediately from 126 to 70 mm. Hg. There was no immediate effect on the carotid systolic pressure, and it remained within the limits of the normal range, until *Oct. 14*, when the aorta just above the origin of both main renal arteries was moderately constricted by another clamp. The femoral mean pressure was immediately reduced slightly from 95 to 80 mm. Hg. There was no immediate effect on the carotid systolic pressure, but in several days it rose to 217 mm. Hg. During the next 7 weeks it fell gradually to normal. On *Dec. 7*, the carotid systolic pressure was 152 mm. Hg, and the femoral mean pressure was 120 mm. Hg.

Occlusion of the aorta by tightening the clamp above the renal arteries, on *Dec. 7, 1937*, and below the renal arteries, on *Mar. 3, 1938*, caused a re-elevation of carotid systolic pressure, but after each clamping it fell gradually to normal.

Apr. 11. Right nephrectomy was also followed by re-elevation of carotid systolic pressure, but again it fell gradually to normal. There was still no indication of renal excretory insufficiency. B.U.N 18.8 mg., Cr. 1.1 mg., CO_2 44.8 volumes per 100 cc. of plasma.

May 4. The left main renal artery was greatly constricted. *May 5.* Carotid systolic pressure rose to 263 mm. Hg, B.U.N. 69.0 mg., Cr. 3.6 mg., CO_2 36.6 volumes per 100 cc. of plasma.

May 7. At 9 a.m. the carotid systolic pressure was 280 mm. Hg, B.U.N. 99.9 mg., Cr. 5.7 mg., CO_2 40.0 volumes per 100 cc. of plasma. The dog was in coma, had convulsions and passed bloody material from the rectum and bladder. During one convulsion the carotid systolic pressure was 330 mm. Hg. Removal of the clamp on the renal artery was followed in about 6 hours by a fall of carotid systolic pressure to 140 mm. Hg, but the animal was in convulsive uremia, B.U.N. 131.3 mg., Cr. 5.1 mg., and died 10 hours after the removal of the clamp.

At autopsy, in the gross, and microscopically, the typical lesions of the malignant phase of this type of hypertension were present in stomach, small and large intestine (except rectum), brain, pituitary, myocardium and epicardium.

No. 3-91: Tan and white hound, female, middle age, 17.0 kg.

Feb. 10 to Mar. 16, 1938. During this pre-operative period the carotid systolic pressure varied between 152 and 172 mm. Hg. *Mar. 16 to Apr. 12.* Constriction of the aorta below the origin of both main renal arteries, on Mar. 16, had no significant effect on the carotid systolic pressure which varied between 150 and

176 mm. Hg, during the normal period. Femoral mean pressure fell from 130 to 60 mm. Hg, immediately after the clamping, but on Apr. 12, it had returned to 110 mm. Hg.

After constriction of the aorta above the origin of both main renal arteries, on *Apr. 12*, carotid systolic pressure rose progressively to a maximum of 280 mm. Hg, on *Apr. 18*. By *June 9*, it had fallen to 192 mm. Hg. Occlusion of the aorta above (June 9) and below (June 27) the main renal arteries was followed by temporary re-elevation of carotid systolic pressure, but, on *Aug. 29*, it was again 190 mm. Hg. On this day the left main renal artery was moderately constricted. Following this, carotid systolic pressure rose to a maximum of 306 mm. Hg. On *Sept. 14*, when it was still 292 mm. Hg, the right main renal artery was greatly constricted.

Sept. 15. The dog appeared obviously ill. The pulse was very rapid and feeble, yet the blood pressure was still high. Carotid systolic pressure was 284 mm. Hg at 9 a.m. B.U.N. 29.3 mg., Cr. 3.0 mg., CO_2 36.2 volumes per 100 cc. of plasma. At 3 p.m. the animal died, immediately after an electrocardiogram was taken.

At autopsy, in the gross, no obvious cause of death was found. The heart weighed 194 gm. and its chambers were greatly dilated. Although the blood creatinine was 3.0 mg. about 6 hours before death, yet the gross anatomical signs of the malignant phase of hypertension, found in other animals, were absent. Microscopically, however, some of the arterioles of the intestine showed hyalinization of the wall. Acute cardiac failure in an animal with early renal excretory insufficiency was considered the most probable cause of death.

No. 3-92: Brown hound, female, middle age, 17.0 kg.

Feb. 10 to Mar. 16. During this pre-operative period the carotid systolic pressure varied between 159 and 172 mm. Hg.

Mar. 16. Moderate constriction of the aorta below the origin of both main renal arteries reduced femoral mean pressure immediately from 120 to 70 mm. Hg, but from Mar. 17 to Apr. 12 the carotid systolic pressure remained unchanged and varied between 156 and 176 mm. Hg. On Apr. 12, it was 156 mm. Hg, and the femoral mean pressure was 90 mm. Hg.

Apr. 12. The aorta was very greatly constricted by a clamp applied just above the origin of both main renal arteries. The femoral mean pressure was reduced immediately to 50 mm. Hg. *Apr. 13 to June 9.* The carotid systolic pressure rose to a maximum of 318 mm. Hg, on Apr. 20, then it fell very gradually to 176 mm. Hg, the upper limit of normal, on June 9.

June 9. The clamp above the origin of both main renal arteries was tightened so as to occlude the aorta. *June 11 to June 27.* The carotid systolic pressure became re-elevated to a maximum of 246 mm. Hg, on June 17, then fell gradually, but was still 220 mm. Hg on June 27.

June 27. The aorta below both main renal arteries was also occluded by tightening of the clamp at this site. *June 28 to Aug. 29.* The carotid systolic

pressure rose again to a maximum of 248 mm. Hg, and then fell gradually to 182 mm. Hg, on Aug. 29.   B.U.N 11.4 mg., Cr. 1.6 mg., CO_2 55.0 volumes per 100 cc. of plasma.

Aug. 29.   The left main renal artery was moderately constricted.   *Aug. 30 to Sept. 14.*   The carotid systolic pressure rose to a maximum of 296 mm. Hg on Sept. 8.   It remained greatly elevated.

Sept. 14.   The right main renal artery was greatly constricted.   *Sept. 15 to Sept. 24.*   The carotid systolic pressure was 280 mm. Hg before the operation on Sept. 14, and it remained elevated at about this level during the entire period following the operation, but the animal progressively developed profound uremia and acidosis until, on *Sept. 28*, the day of death, B.U.N. 225.0 mg., Cr. 11.2 mg. and CO_2 24.1 volumes per 100 cc. of plasma.

At autopsy, in the gross, and microscopically, petechiae and the typical arteriolar lesions of the malignant phase were present in the mucosa and serosa of the stomach, small intestine, large intestine, pancreas, urinary bladder, mesenteric and periaortic lymph nodes, epicardium, brain and diaphragmatic muscle.

No. 3-57: Collie, male, young, 10.0 kg.

Aug. 31 to Oct. 20, 1937.   During the pre-operative period the carotid systolic pressure varied between 120 and 135 mm. Hg.

Oct. 20.   The aorta below the origin of both main renal arteries was very greatly constricted.   The femoral mean pressure was reduced immediately from 120 to 48 mm. Hg.   *Oct. 21 to Dec. 7.*   During this period the carotid systolic pressure varied between 144 and 150 mm. Hg, which was well within normal limits for this animal.   The femoral mean pressure gradually rose to 120 mm. Hg. On Dec. 7, the carotid systolic pressure was 146 mm. Hg.

Dec. 7.   The aorta above the origin of both main renal arteries was greatly constricted by means of another clamp.   This reduced the femoral mean pressure only slightly, to 100 mm. Hg, although the plate was screwed down almost as far as it would go.   *Dec. 8 to Dec. 24.*   The carotid systolic pressure rose in a few days to a maximum of 240 mm. Hg.   At the same time even the femoral mean pressure rose above normal to 175 mm. Hg.   On Dec. 24, the carotid systolic pressure was 236 mm. Hg, the carotid mean pressure was 195 mm. Hg, and the femoral mean pressure was 160 mm. Hg.   *Dec. 26.*   The animal was found dead.   At autopsy, the cause of death was found to have been intra-abdominal hemorrhage caused by erosion of the aorta by the clamp above the origin of both main renal arteries.

No. 3-89: Black and white, short haired mongrel, male, middle age, 9.0 kg.

Jan. 29 to Mar. 1, 1938.   During the pre-operative period the carotid systolic pressure varied between 134 and 160 mm. Hg, and the femoral mean pressure varied between 120 and 145 mm. Hg.

Mar. 2.   Right nephrectomy.   *Mar. 3 to May 5.*   Carotid systolic pressure remained within normal limits.

May 6. The aorta below the origin of both main renal arteries was greatly constricted. The femoral mean pressure was immediately reduced from 120 to 60 mm. Hg. During the next 3 days, there was no elevation of the systolic blood pressure, which was 152, 146 and 154 mm. Hg on successive days. *May 10.* The animal was found dead. The cause of death was not determined.
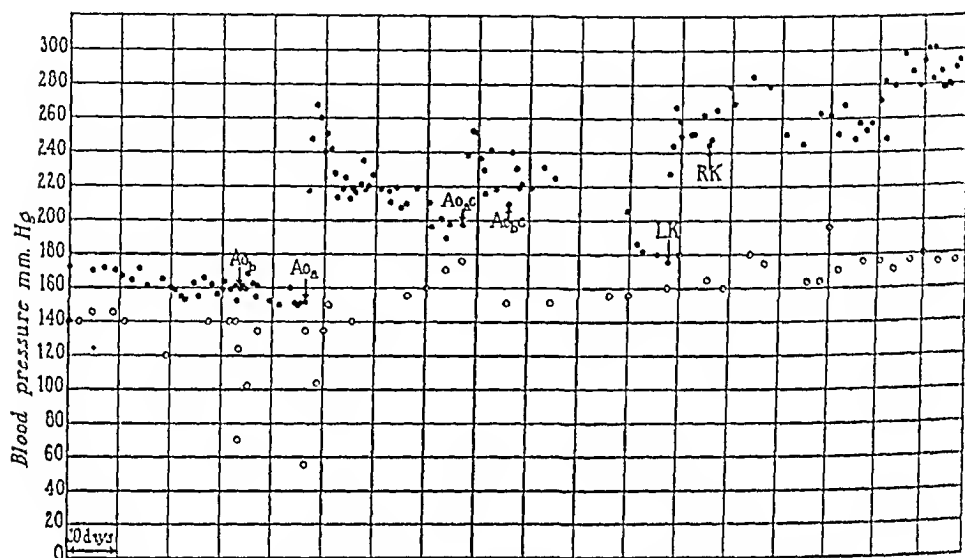
TEXT-FIG. 2. No. 3-83: Doberman pinscher, female, young, 18 kg.

Ao_b, abdominal aorta just below the origin of both main renal arteries greatly constricted by a clamp. Ao_a, abdominal aorta just above the origin of both main renal arteries greatly constricted by a clamp. Ao_ac, abdominal aorta above the main renal arteries occluded by tightening the clamp. Ao_bc, abdominal aorta below the main renal arteries occluded by tightening the clamp. LK, left main renal artery greatly constricted by a clamp. RK, right main renal artery greatly constricted by a clamp.

The figures for blood pressure which appear as solid black circles represent the carotid systolic pressure determined by the van Leersum carotid loop method (3, 94). The figures which appear as open circles represent femoral mean pressure determined by the direct method.

The Effect of Constriction of the Aorta below the Origin of the Main Renal Arteries Followed Later by Constriction of the Aorta above the Renal Arteries and Finally Constriction of the Main Renal Arteries

Constriction of the main renal arteries was finally necessary to effect persistent elevation of blood pressure in some animals with the

aorta constricted or even occluded below, and later above, the origin of the main renal arteries. On dog 3-83 (Text-fig. 2), the constriction of the aorta below the origin of the main renal arteries had no significant effect on the carotid systolic pressure. When this was followed by constriction of the aorta above the site of origin of both main renal arteries, there was a prompt and significant rise of carotid systolic as well as mean pressure. Carotid systolic pressure then fell gradually over a period of about 2 months, but did not quite return to the normal level. Occlusion of the aorta first above and, about 3 weeks later, also below the origin of the main renal arteries, resulted in a re-elevation of carotid blood pressure which again did not persist. In about 3 months the carotid systolic pressure had gradually returned to slightly above the normal level for this dog. Moderate constriction of first the left and, a few days later, the right renal artery, resulted in great elevation of carotid systolic and carotid mean pressure, and even the femoral mean pressure became and has remained significantly elevated above the normal, despite the occlusion of the abdominal aorta at two sites. The animal is still alive about 4 months after the constriction of the renal arteries, the blood pressure in the carotid loop is still greatly elevated (Text-fig. 2), and there is no chemical evidence of renal excretory insufficiency.

The Effect of Constriction of the Abdominal Aorta Just above the Origin of the Main Renal Arteries on the Blood Pressure of Animals with the Main Renal Arteries Previously Constricted

In a few animals in which both main renal arteries had been constricted, but in which, after a variable period of hypertension, the blood pressure had returned to a lower level, constriction of the abdominal aorta above the origin of both main renal arteries resulted in a re-elevation of blood pressure. Two animals (Nos. 2-48, 3-03) died of hemorrhage due to erosion of the aorta. One of these survived 8 days, the other 21 days. During the period of survival both animals showed a persistent elevation of carotid systolic pressure.

One animal (No. 2-31), with renal arteries previously constricted, died of uremia after the abdominal aorta just above the renal arteries was constricted. In this dog the blood pressure had returned to a lower level, but was still elevated, and renal excretory function was

normal.   During the period of survival following constriction of the aorta above the main renal arteries, the carotid systolic pressure became greatly elevated.

In one animal (No. 1-40) with the aorta constricted above the origin of the main renal arteries, after previous constriction of both main renal arteries had failed to keep the blood pressure at a high level, the exact cause of death was not determined.   After constriction of the aorta, the period of survival was short, but the carotid systolic blood pressure rose to a very high level.

No. 2-48: Shepherd, female, middle age, 18.0 kg.

Jan. 15, 1936, to Mar. 3, 1937.   During the normal period of more than 1 year, the carotid systolic pressure varied between 120 and 156 mm. Hg.   During the last 6 months of this period, it never was higher than 138 mm. Hg, and most frequently it varied between 120 and 130 mm. Hg.   On Mar. 3, 1937, the carotid systolic pressure was 128 mm. Hg.

Mar. 3.   The right main renal artery was moderately constricted.   *Mar. 4 to Mar. 31.*   During this period the carotid systolic pressure slowly reached a maximum of 196 mm. Hg, and then fell rather abruptly to 166 mm. Hg.

Mar. 31.   The left main renal artery was moderately constricted by a clamp.   *Apr. 1 to Sept. 22.*   During this time the carotid systolic pressure rose in a few days to a maximum of 210 mm. Hg, and then fell gradually to 168 mm. Hg.

Sept. 22.   B.U.N 17.9 mg., Cr. 1.5 mg., CO_2 48.2 volumes per 100 cc. of plasma. The aorta was very greatly constricted by a clamp applied just above the origin of the main renal arteries.   The femoral mean pressure was reduced immediately from 140 to 50 mm. Hg.   *Sept. 23 to Sept. 30.*   During this period the carotid systolic pressure rose to 292 mm. Hg, on Sept. 25, and remained above 240 mm. Hg until death.   *Oct. 1.*   The animal was found dead.   At autopsy, death was found to have been due to erosion of the aorta by the clamp.

No. 3-03: Hound, black and white, middle age, female, 15.0 kg.

Dec. 1, 1936, to Jan. 12, 1937.   During the normal period the carotid systolic pressure varied between 160 and 170 mm. Hg, and the femoral mean pressure varied between 115 and 145 mm. Hg.   During the last weeks of this period the femoral mean pressure was never higher than 130 mm. Hg.   *Jan. 12, 1937, to Sept. 8, 1938.*   Laminectomy and section of anterior nerve roots from sixth dorsal to second lumbar, inclusive, were performed on Jan. 12, 1937.   This had no significant effect on blood pressure.   On Jan. 26, 1938, and Feb. 3, the left and right main renal arteries, respectively, were moderately constricted.   On Apr. 22 and June 30, the left and right main renal arteries, respectively, were occluded. During the intervals between the operations the carotid systolic pressure first rose to high levels (maximum 290 mm. Hg), but always fell again.   On Sept. 8,

1938, left nephrectomy was performed. From *Sept. 9 to Oct. 26*, despite the occluded right main renal artery, and absence of left kidney, no chemical evidence of renal insufficiency developed. During this period carotid systolic pressure was well over 200 mm. Hg for about 3 weeks, and then fell to 190 mm. Hg.

Oct. 27. The aorta was moderately constricted by a clamp applied just above the origin of both main renal arteries. The femoral mean pressure was reduced immediately from 160 to 80 mm. Hg. *Oct. 27 to Nov. 17.* The carotid systolic pressure rose to 240 mm. Hg, and remained around this level until Nov. 15, on which day the last determination was made. The femoral mean pressure, despite the clamped aorta, gradually rose to 180 mm. Hg, which was well above the normal range when the aorta was wide open. The animal was seen at 9 a.m. on Nov. 11, and seemed perfectly well. At 10 a.m. it was found dead. At autopsy, the cause of death was found to have been massive intra-abdominal hemorrhage due to erosion of the aorta by the clamp.

No. 2-31: Black mongrel terrier, female, young, 20.4 kg.

Nov. 20 to Dec. 13, 1935. During the normal period the carotid systolic pressure varied between 142 and 160 mm. Hg.

Dec. 13. The right main renal artery was moderately constricted by a clamp. *Dec. 14, 1935, to Jan. 7, 1936.* The carotid systolic pressure rose to a maximum of 232 mm. Hg, on Dec. 20, 1935, and then fell gradually to 166 mm. Hg on Jan. 7, 1936.

Jan. 7, 1936. The left main renal artery was moderately constricted. *Jan. 8, 1936, to Jan. 18, 1938.* During more than 2 years the carotid systolic pressure varied between 190 and 230 mm. Hg. On Jan. 15, 1938, it was 202 mm. Hg.

Jan. 18, 1938. The aorta was very greatly constricted by a clamp applied just above the origin of both main renal arteries. The femoral mean pressure was reduced immediately from 160 to 70 mm. Hg. B.U.N. 24.0 mg., Cr. 1.3 mg., CO_2 48.2 per 100 cc. of plasma. *Jan. 20.* B.U.N. 64.0 mg., Cr. 4.9 mg. per 100 cc. of plasma. The carotid systolic pressure was 255 mm. Hg. *Jan. 21.* The animal was found dead. At autopsy, petechiae were found in the mucosa of small and large intestine and of urinary bladder. Microscopically, the arterioles were hyalinized and some were necrotic. Much of the hemorrhage was of capillary origin.

No. 1-40: Black and white, short haired mongrel, female, 12.8 kg.

Jan. 5, 1934, to Mar. 28, 1935. During this normal period of more than 1 year the carotid systolic pressure varied between 134 and 162 mm. Hg.

Mar. 29, 1935, to Sept. 23, 1937. During this period of more than 2 years, first one and then the other main renal artery was constricted and finally occluded. After each operation the carotid systolic pressure rose and at times reached a maximum of 290 mm. Hg. However, on *Sept. 24, 1937*, carotid systolic pressure had returned to 170 mm. Hg, and there was no chemical evidence of renal excretory

insufficiency. B.U.N. 10.0 mg., Cr. 1.2 mg., CO_2 49.0 volumes per 100 cc. (plasma.

Sept. 24. The aorta above the origin of both main renal arteries was greatl constricted. The femoral mean pressure was reduced immediately from 150 t(60 mm. Hg. *Sept. 25.* The carotid systolic pressure was 236 mm. Hg, B.U.N 26.0 mg., Cr. 1.3 mg., CO_2 34.8 volumes per 100 cc. of plasma. *Sept. 26.* The carotid systolic pressure was 270 mm. Hg. The animal died suddenly soon after the blood pressure was determined. The blood was not examined chemically on this day, so that the presence of uremia cannot be excluded, but there were no anatomical signs of uremia associated with hypertension. The heart chambers were greatly dilated. The lungs were edematous and hyperemic. Acute cardiac failure was regarded as the most probable cause of death.

The Effect of Constriction of the Abdominal Aorta in a Bilaterally Nephrectomized Animal

In one animal (No. 4-13) the aorta was constricted at the same time that the second kidney was removed. The animal survived 2 days. During this time the carotid systolic pressure did not become elevated, although azotemia of a high degree, without convulsions, developed before death.

No. 4-13: Black and white, short haired, mongrel, female, 13.6 kg.

May 12 to Oct. 26, 1938. During the pre-operative period the carotid systolic pressure varied between 142 and 162 mm. Hg. The femoral mean pressure varied between 120 and 145 mm. Hg.

Oct. 26. Right nephrectomy. *Oct. 27 to Nov. 8.* During this period the blood pressure did not become elevated. Carotid systolic pressure varied between 136 and 160 mm. Hg, and the femoral mean pressure varied between 125 and 140 mm. Hg. *Nov. 7.* B.U.N. 12.0 mg., Cr. 1.7 mg., CO_2 50.1 volumes per 100 cc. of plasma.

Nov. 8. Left nephrectomy. A clamp was also applied to the aorta just above the site of the left main renal artery. The aorta was greatly constricted. The femoral mean pressure was reduced immediately from 140 to 55 mm. Hg. The operations were performed under local anesthesia (procaine) with a previous hypodermic injection of morphine and atropine. Immediately after the operation the animal appeared perfectly normal and was able to eat and drink as if nothing had been done to it. For the next 2 days it continued to appear well. It drank water, but refused food. There was no immediate effect on the carotid systolic or carotid mean pressure which remained unchanged at 146 mm. Hg immediately after the clamping. Several determinations of blood pressure were made during the day of the operation and during the survival period of 2 days. At no time was the pressure higher than 156 mm. Hg, which was within the normal limits,

although the animal developed marked azotemia. *Nov. 9.* Carotid systolic pressure 142 mm. Hg at 9 a.m. and 114 mm. Hg at 4 p.m. *Nov. 10.* B.U.N. 123.0 mg., Cr. 8.4 mg., CO_2 60.4 volumes per 100 cc. of plasma. The carotid systolic pressure was 74 mm. Hg at 9 a.m. The animal appeared moribund. It was found dead at 9.15 a.m.

This animal developed marked azotemia, without convulsions or hypertension. At autopsy, there were no gross petechiae or microscopic lesions in the arterioles such as have been found commonly in our animals with convulsive uremia complicating hypertension (9). There was also one other striking difference. In this animal, even terminally, the CO_2 capacity was higher than normal, whereas in animals with uremia complicating hypertension, even terminally, it is usually lower than normal.

The Effect of Constriction of the Thoracic Aorta

Several attempts to constrict the aorta at various levels within the thorax were without conclusive results, due to technical difficulties. Acute dilatation of the heart, intrathoracic hemorrhage due to erosion of the aorta by the clamp, pneumonia with empyema, or hydrothorax with pulmonary atelectasis caused the early death of these animals. None of the animals lived long enough without complications to permit any conclusion about the effects on blood pressure of persistent intrathoracic constriction of the aorta. This part of the study is being continued.

DISCUSSION AND SUMMARY

Constriction of the aorta just above the origin of both main renal arteries invariably resulted in elevation of the carotid systolic and carotid mean pressure. The hypertension was not immediate, but developed in about the same time as after constriction of the main renal arteries (3). Constriction of the aorta just below the origin of both main renal arteries had no significant effect on the carotid systolic or carotid mean pressure. Since these results were first reported (1), Rytand (88, 89) has shown by an indirect method, namely, the demonstration of the development of cardiac hypertrophy, that hypertension in the upper part of the body can be produced in the rat by constriction of the aorta just above the origin of both main renal arteries.

The immediate effect of constriction of the aorta either below or above the main renal arteries is a fall of blood pressure (femoral mean

pressure) below the site of the clamp, the extent of the fall being directly dependent upon the degree of constriction of the aorta. Of particular interest is the eventual elevation of the femoral mean pressure above the normal in some animals with the aorta constricted or even occluded above the origin of the main renal arteries. This was most pronounced and persistent in those animals in which, in addition, the aorta below the origin of the renal arteries, and, in some animals, the main renal arteries, also were constricted. The most important factors which determined this elevation of blood pressure in the lower part of the body were probably increased flow of blood into the vascular bed below the clamp and peripheral vasoconstriction of renal and humoral origin, as in the case of the hypertension produced by constriction of the main renal arteries alone (2–86). Although elevation of the carotid systolic or carotid mean pressure occurred invariably within 24 to 48 hours after the constriction of the aorta above the site of origin of both main renal arteries, yet there was a tendency, after a variable period, for the elevated blood pressure to become lower or even to drop to the original level. Increased constriction, and finally occlusion of the aorta, above the origin of the main renal arteries, and even constriction or occlusion of the aorta below the renal arteries, in addition, failed to induce hypertension that persisted for a long time at a high level. In order to produce this effect, it was necessary to constrict the main renal arteries as well.

The possible explanation of the failure of the hypertension to persist for a long time after constriction of the aorta alone, is that the initial ischemia of the kidneys disappeared due to the improvement of the blood flow through the kidneys as a result of (a) the increase of the natural accessory circulation to the kidneys; (b) the increased blood pressure above the site of the clamp and consequent increased flow of blood into the part of the aorta below the clamp; (c) increased pressure below the site of the clamp due, in great part, to peripheral vasoconstriction, and in part to the increased inflow of blood into the lower part of the body through the aorta and collateral channels. For the dog, this method is not necessary for the production of persistent hypertension. Constriction of the main renal arteries is easily performed and is effective for the production of generalized hypertension (2–11). However, constriction of the aorta in addition

to constriction of the renal arteries results in greatly elevated persistent hypertension. Constriction of the aorta alone above the origin of the main renal arteries would be useful in the dog only for the production of relatively short periods of hypertension in the upper part of the body. For small animals it may be a more effective and useful method. In the dog, the only technical difficulty encountered was the erosion of the wall of the aorta by the clamp. This may not occur in small animals. In previous studies (2–11) that have dealt with the constriction of the main renal arteries, this accident rarely occurred.

When the constriction of the aorta above the origin of the main renal arteries was of moderate degree, or was gradually made very great, the resultant hypertension was not accompanied by impairment of renal excretory function, as determined by urea clearance or by the quantity of urea, creatinine or non-protein nitrogen in the blood, the benign phase of hypertension (3). When the constriction of the aorta was suddenly made very great, impairment of the renal excretory function usually followed, and the animal developed fatal convulsive uremia and characteristic vascular lesions, the malignant phase of hypertension (9). These facts are all indicative of the renal origin of the hypertension which results from the constriction of the aorta just above the origin of both main renal arteries.

Hypertension did not persist for a sufficiently long time to permit any conclusive comparison between the effect of the high and low pressures on the structure of the vascular system, above and below the site of the clamp, respectively. During the period of survival of these animals, no significant differences were observed between the appearance of the vascular system of the upper part of the body and that of the lower part of the body, and significant cardiac hypertrophy did not develop. In the aorta and large arteries, intimal arteriosclerosis was not observed. In the aorta of one old animal several small plaques of calcification were found in the media, but these were present in the portion of the aorta below, as well as above the clamp, and they were no larger or more abundant than were observed in some old dogs with normal blood pressure. Dogs 3-50 and 3-83, that are still alive, with very high blood pressure above the site of the aortic clamps, and relatively low pressure (though greater than

normal) below the site of the aortic clamps, will be valuable for the determination of possible differences between the effects of the two levels of blood pressure in the large and small blood vessels. In these dogs also, it will be possible to determine the effect of the persistently high blood pressure on the myocardium.

The possible application of the results of this study to the problem of the pathogenesis of human eclampsia is mentioned here for consideration. Since this condition occurs in pregnancy only at a time when the uterus is greatly enlarged, it is at least possible that the mass may press on the aorta or both main renal arteries sufficiently to produce renal ischemia. The suddenness with which the uremic convulsive phase of eclampsia develops is in keeping with this idea. In the dog, an aggravating effect of pregnancy on an already established hypertension has not been noted. As a matter of fact, most of the hypertensive dogs that have become pregnant, have shown a slight or moderate fall, rather than an increased rise of pressure. Since the dog stands with the body in a horizontal position, and does not lie on its back, pressure of the pregnant uterus on the aorta and blood vessels is less than in human beings who stand erect and frequently lie on their backs. The soundness of this suggestion could be tested by placing pregnant women, in the early stage of eclampsia, in a position which could relieve possible pressure on the aorta and main renal arteries.

A possible explanation of the fall of pressure in the pregnant hypertensive dogs is the compensatory effect of the normal kidneys of the pups, as in the case of an animal with one main renal artery constricted and the other kidney normal. As has been shown (3, 31, 72), the presence of one normal kidney in an animal hypertensive due to constriction of the other main renal artery, results, after a variable period, in a return of the blood pressure to normal. How the normal kidney acts to produce this effect is not known.

CONCLUSIONS

Constriction of the abdominal aorta just above the site of origin of both main renal arteries has little or no immediate effect on the blood pressure above the site of the clamp, (carotid systolic or mean pressure), but in about 24 hours, hypertension develops. Below the site

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STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

II. PATHOGENESIS OF THE DISEASE IN THE GUINEA PIG

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PLATE 40

In the first paper of this series (1), the histopathology of equine encephalomyelitis in the guinea pig was described and discussed in detail. The virus injected peripherally readily invades the blood stream and then infects the central nervous system. The mode of entrance of the virus into the nervous system from an injection site at the periphery is not clear.

Hurst (2), studying the guinea pig, believed that the local peripheral nerves are not involved in the pathogenesis. He suggested that virus is deposited from the blood on the olfactory mucosa, whence it travels to the subdural space by means of the perineural lymphatics. The recent work of Yoffey and Drinker (3) makes such an explanation improbable. Hurst also suggested the passage of virus from the blood across the endothelium of the cerebral blood vessels (in his phrase, a "growth through" the hematoencephalic barrier).

Sabin and Olitsky (4) believed that, in contrast to the behavior in mice, the virus in the guinea pig passes directly across the vascular endothelium, since in this animal the lesions bear a definite relation to blood vessels. With the Western strain of the virus, Larsell, Haring, and Meyer (5) similarly suggested a direct passage across the blood vessels, also on the basis of the perivascular nature of the lesions. The validity of this evidence will be treated in the discussion.

Because of the lack of unequivocal data, the problem was reinvestigated. Topographical analysis of early cases seemed the most promising method. This method depends on the assumption that the earliest localization of the virus will produce the first lesions. Although, as is well known, virus may be present without causing tissue damage, an area with a demonstrable typical lesion presumably has harbored the virus for a longer period than any area without lesions.

By charting all the lesions in a brain the earliest localizations can thus be determined.

Method

Since topographical analysis would be valueless when the encephalitis is fully developed, it was essential to secure very early cases for study.   To ensure the presence of lesions before the brains were serially sectioned, a method of vital staining was used, modified from that of McClellan and Goodpasture (6).   The following procedure was used in guinea pigs.   1 to 2 hours before the animal is to be sacrificed, 2 to 3 cc. of 2 per cent trypan blue are injected intravenously.   It is usually desirable to administer an additional 1 cc. subcutaneously the day previously.   The intravenous injection usually prostrates the animal which, after 1 to 2 hours, is killed with chloroform.   The nervous system is then perfused with formalin through the aorta under physiological pressure.   When the brain is removed, lesions are clearly outlined in blue against the colorless background.   The entire success of the method depends on securing a good perfusion.   Otherwise even the normal portions of the brain may be colored, due to dye contained within the blood vessels.

With this method, animals were killed at various intervals after inoculation.   The nerve tissue was imbedded in paraffin and sectioned serially at 15 microns.   Every 15th or 20th section was mounted and stained.

Two strains of virus were used.   One was isolated in 1937, and the 2nd, 3rd, and 4th subinoculations in guinea pigs were employed.   The 2nd was isolated in 1938, and the 1st guinea pig subinoculation was used.   The results of the two strains were indistinguishable.

OBSERVATIONS

Observations on 9 selected animals, none of which showed any clinical signs of disease at the time of sacrifice, are presented in detail.   Even a cursory survey of the material shows that, with a constant mode of inoculation, the lesions are never the same in any two cases.   In the accompanying figures are charted the lesions which appear on the surface.   All those which are hidden from view in the interior of the brain are described in the accompanying text.   Summaries are presented in Table I and should be consulted in conjunction with the charts.   For a detailed description of the anatomical structures mentioned below, reference may be made to standard works on the nervous system of rodents (7).

No. 1.   Inoculation into both hind pads.   Sacrificed after 58 hours.   Every 15th section mounted and stained.—Both olfactory bulbs contain a few small discrete

TABLE I

Guinea pig No.	Route of inoculation	Killed (time after inocu-lation)	Summary of lesions
		hrs.	
1	Hind pads	58	Small discrete lesions scattered over the frontal part of the brain, involving the olfactory and neocortical portions about equally. Similar lesions present in the cerebellum. Subcortical centers intact
2	" "	$57\frac{3}{4}$	Scattered lesions present in neocortex and cerebellar cortex, as well as in a few unrelated subcortical centers
3	" "	$58\frac{3}{4}$	Scattered lesions involving portions of the olfactory brain and unrelated areas in the neocortex. Subcortical lesions situated only in the intermediate auditory centers; lowest, primary auditory nuclei normal
4	" "	$62\frac{1}{4}$	Cerebral lesions restricted to extensive involvement of the olfactory regions, without affecting the hippocampus. Isolated lesions in the cerebellar cortex
5	" "	$58\frac{1}{2}$	Inflammatory involvement primarily of the lower and higher visual centers of both sides, more severe on the left. Most of the thalamus, including the non-visual portions, affected, as well as certain auditory centers. Parts of the olfactory pathways, as well as the basal ganglia, also injured
6	Intranasal	67	Widespread lesions in lower olfactory connections; but circumscribed, unrelated lesions also present in neocortical areas, the caudate nucleus, the cerebellum, and the mid-brain
7	Intraocular (right)	66	Involvement of right anterior olfactory regions, and of scattered neocortical areas. Thalamus and subcortical centers intact
8	" "	58	Involvement of entire optic pathway of both sides, from the chiasm to the cortex, the left side more than the right. Lesions extend to contiguous areas which are functionally independent. Isolated lesions present in the left claustrum and pons, regions independent of each other and of the affected portions elsewhere. Certain intermediate acoustic centers affected
9	" "	$58\frac{1}{2}$	A few scattered lesions in the intermediate olfactory centers and in the neocortex. Olfactory bulbs normal. Lower visual centers corresponding to the inoculated eye show very early involvement, but of much less intensity than the cortex
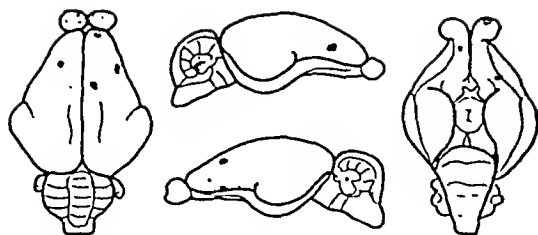
TEXT-FIG. 1. Guinea pig 1.
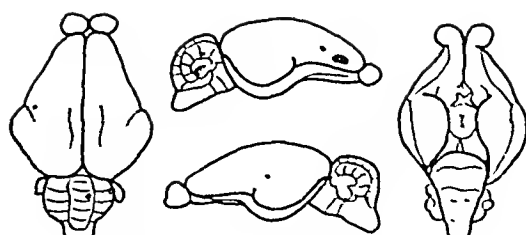
TEXT-FIG. 2. Guinea pig 2.
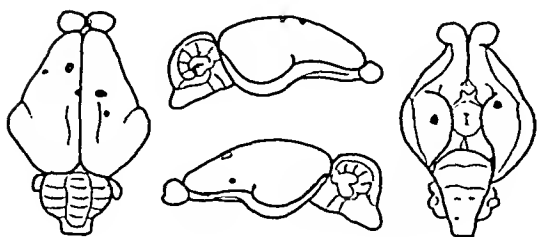
TEXT-FIG. 3. Guinea pig 3.
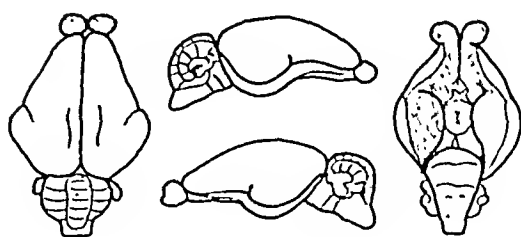
TEXT-FIG. 4. Guinea pig 4.
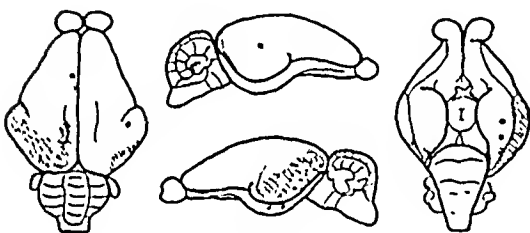
TEXT-FIG. 5. Guinea pig 5.
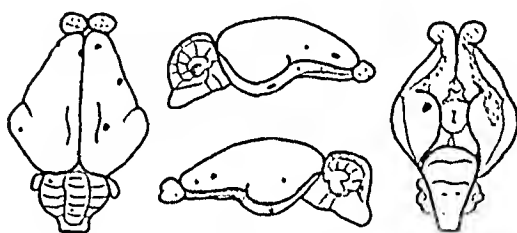
TEXT-FIG. 6. Guinea pig 6.
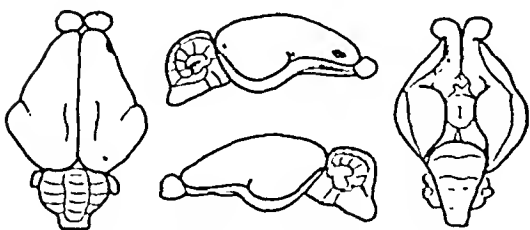
TEXT-FIG. 7. Guinea pig 7.
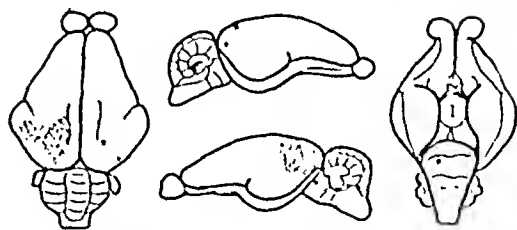
TEXT-FIG. 8. Guinea pig 8.
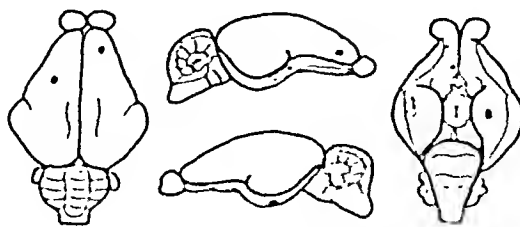
TEXT-FIG. 9. Guinea pig 9.

lesions. In the remainder of the olfactory brain there are only two other lesions, one in the anterior olfactory nucleus on the right, the other astride the rhinal fissure on the left, involving the neo- and olfactory cortex about equally on either side of the fissure. There are other lesions in the neocortex, as indicated in the chart, affecting the orbital, precentral, postcentral, and insular areas. There are no concealed lesions; the hippocampus, basal ganglia, thalamus, midbrain, pons, and medulla are entirely normal, as is the spinal cord. In the cerebellum there are a few sharp foci, two of which, in the flocculi, show exquisite bilateral symmetry.

No. 2. Inoculation into both hind pads. Sacrificed after 57¾ hours. Every 15th section mounted and stained.—No part of the olfactory system is involved. In the neocortex lesions are present in the right postcentral area, involving the insular cortex to some extent, and the right parietal region. There is a small focus of low intensity in the left temporal cortex. Two isolated lesions are present in the cerebellar cortex. Several lesions are present in lower centers, not indicated in the chart. There is bilateral ependymitis with, in one area, a collection of polymorphonuclear leucocytes impinging on the ventricle. The left caudate nucleus also contains a small but intense focus of inflammation. The right medial geniculate body and the right inferior colliculus contain early lesions, but the acoustic tubercles and intermediate centers are entirely normal. No other lesions are present in any portion of the brain or cord. In the meninges are two small areas, one in the left temporal cortex and one at the base at the level of the posterior thalamus, where there is some mononuclear infiltration, slight in both degree and extent. These meningeal areas are not related to regions of parenchymatous inflammation.

No. 3. Inoculation into both hind pads. Animals sacrificed 58¾ hours later. Every 15th section mounted and stained.—In the entire olfactory system there are only three lesions, one in the medial portion of the right bulb and one in the entorhinal region of either side. The intermediate centers are intact, as is the hippocampus. Most of the lesions are situated in the neocortex where the left precentral and parietal, and the limbic or infraradiar areas are involved. On the right the lesions affect the parietal and the most anterior portion of the striate areas. Hidden from view are early lesions in the right inferior colliculus and right medial geniculate body. No other portion of the thalamus shows any injury. The lateral geniculate, sending fibers to the striate cortex, and the anterior nuclei, which send afferents to the limbic area, are intact. The medullary acoustic centers, along with the entire medulla, cerebellum, and cord, are normal.

No. 4. Inoculation into both hind pads. Sacrificed after 62¼ hours. Every 20th section mounted and stained.—The olfactory bulbs of both sides contain numerous minute foci of polymorphonuclear leucocytes. There is in addition much more extravasation of blood than is usually seen. The olfactory centers at the base of the brain are affected in very widespread fashion. On the right practically the entire extent is involved by innumerable small foci, sometimes dicrete, sometimes confluent, of varying degrees of intensity. On the left the process is similar but essentially limited to the anterior pyriform cortex, sparing the tuberculum

olfactorium. The process extends inward to a slight extent, to include the amygdala and the nucleus accumbens. Although the presubiculum is damaged, the involvement does not include the hippocampus. For the most part the inflammatory changes are sharply limited dorsally by the rhinal fissure, but in a few areas there is a very slight extension into the adjacent neocortex. The only other sign of encephalitis in the entire brain is the presence of three isolated discrete foci of inflammation in the cerebellar cortex. Thalamus, midbrain, pons, and medulla are intact.

No. 5. Inoculation into both hind pads. Sacrificed after 58½ hours. Every 20th section mounted and stained.—The olfactory bulbs are intact. There is a minute lesion in the left olfactory crus, in the dorsal part of the left anterior olfactory nucleus adjacent to the orbital cortex. There are also two discrete foci of destruction in the left entorhinal cortex. Apart from a single isolated focus in the left precentral area, the other neocortical lesions are restricted to the striate, temporal, and occipital areas. On the right there is a single circumscribed lesion in the visual area, and a more diffuse region of injury more posteriorly, which overflows into the inferior temporal cortex and the subiculum. On the left, however, the damage is quite extensive, involving the greater part of the visual and posterior temporal cortex, and also overflowing into the subiculum. It will be noticed that the chiasm is intact, but the optic tracts exhibit small discrete lesions, more on the left.

Hidden from view there is rather widespread damage to the telencephalon and diencephalon. Thus, the left caudate nucleus contains a small focal collection of polymorphonuclear leucocytes. The left putamen and globus pallidus and the amygdala more posteriorly also contain early lesions. In the thalamus there are inflammatory changes involving the anterior group, the lateral, and the ventral nuclei, on the left. The medial as well as the lateral geniculate bodies of both sides are severely injured, as well as the pretectal regions and the posterior nuclei. There is a much milder involvement of the medial nuclei of the thalamus, the subthalamus, and portions of the hypothalamus. The changes in the superior colliculi are well marked; those in the inferior colliculi are very mild, with the right somewhat greater than the left.

The white matter of the hemispheres, including the thalamic peduncles, shows very numerous glial nodules, and some cuffing of the blood vessels. The cerebellum, pons, and medulla, as well as the hippocampus, are normal. There are no changes in the medullary acoustic centers. The spinal cord is normal.

No. 6. Intranasal instillation, both nostrils. Sacrificed at 67 hours. Every 20th section mounted and stained.—The maximum involvement affects the basal olfactory centers. The bulbs are the seat of numerous small lesions, which, on the left, continue backward in a rather diffuse fashion, including the anterior olfactory nucleus and pyriform cortex. The tuberculum olfactorium is bilaterally spared. The lesions diminish in intensity posteriorly and disappear in the position marked. On the left the process is much less severe, only the anterior pyriform cortex being affected. More posteriorly is an isolated lesion as marked. The

hippocampus is entirely spared. In the neocortex there are numerous discrete circumscribed lesions situated in diverse architectonic areas—precentral, postcentral, parietal, insular, temporal, and striate. The cerebellar cortex also displays two isolated lesions.

Hidden from view there is a mild degree of ependymitis of the left lateral ventricle. The left caudate nucleus contains a small inflammatory focus. The right lateral geniculate body, which sends fibers to the striate cortex, is intact; but the right superior colliculus, which merely receives from the cortex without sending, shows very early changes. Both inferior colliculi show extensive but very early involvement, the right somewhat more than the left. But the medial geniculate bodies, as well as all the medullary acoustic centers, are completely normal.

No. 7. Intraocular inoculation into the right eye. Sacrificed after 66 hours. Every 20th section mounted and stained.—The right olfactory bulb shows a few small scattered lesions. On the same side the anterior olfactory nucleus and the anterior pyriform lobe show well marked inflammatory changes. In the neopallium there are three discrete lesions, one in the right postcentral cortex, and one in the right temporal cortex. There is in addition a small focus of neuronal degeneration in the right occipital cortex. The thalamus and other subcortical centers are entirely normal.

No. 8. Intraocular inoculation into the right eye. Animal sacrificed 58 hours later. Every 20th section mounted and stained.—The olfactory bulbs and entire anterior portion of the brain, including neocortex, olfactory cortex, and anterior basal ganglia, are entirely free of lesions. The first lesions are seen in the optic chiasm and optic tracts, where the left is more severely affected than the right, although both contain glial and leucocytic foci. The hypothalamus and olfactory regions adjacent to the ascending optic tract on the left show well marked inflammatory reaction. The surface lesions are restricted to the striate and occipital cortex on the left, where the involvement is widespread. On the right, there are only two discrete focal lesions in the striate cortex. In addition there are seen lesions in the left midbrain (nucleus of the lateral lemniscus) and in the right pons, both reaching the surface of the brain. Hidden from view are lesions in both lateral geniculate bodies and both superior colliculi, the left being more severely damaged than the right. Both medial geniculates are involved, but the inferior colliculi are intact. The damage to the midbrain and posterior thalamus is not restricted to the nuclei mentioned, but tends to extend medially in a somewhat diffuse fashion. There is an isolated circumscribed lesion in the left claustrum at the level of the infundibulum. The medulla, cerebellum, and cord are entirely normal. The medullary acoustic centers show no change.

No. 9. Intraocular inoculation into the right eye. Sacrificed after 58½ hours. Every 20th section mounted and stained.—The olfactory bulbs are free of lesions although there are discrete scattered lesions more posteriorly in the rhinencephalon, namely, small early foci in the right anterior pyriform cortex and the right tuberculum olfactorium. More posteriorly there is a well circumscribed

focus in the left entorhinal cortex and a more diffuse area of involvement in the comparable region on the right side. In the neocortex there are lesions in the right precentral and insular areas, and also in the right postcentral and parietal areas. In the subcortical centers, hidden from view, the left lateral geniculate and the left anterior colliculus show extremely early but very definite lesions. The remainder of the thalamus is entirely intact, as is the hippocampus, as well as all other portions of the brain.

General Features

It is of interest to observe whether virus lesions are isolated or whether they tend to involve systems. Of the 9 cases presented, in 4 there is definite system involvement, although of different sense modalities: the olfactory pathway in Nos. 4 and 6, and the visual pathway in Nos. 5 and 8. It is noteworthy that different routes of inoculation were utilized. In line with this, it is seen that the same route of inoculation can, in different instances, produce totally different distribution of lesions (as No. 8 contrasted with No. 9, both with intraocular injections; or, with pad inoculation, No. 4 contrasted with No. 2). On the other hand, as already mentioned, different modes of inoculation can produce substantially the same result.

Apart from the occasional system involvement, there should be noted the frequency of discrete scattered lesions in the cortex, of the hemispheres, the cerebellum, and the olfactory brain. The virus appears to attack the brain not only with reference to nerve paths, but also independently of known system connections.

It is of further interest that although the cortex is the site of predilection, subcortical centers may also be affected at times. Thus, various regions in the basal ganglia, thalamus, hypothalamus, and midbrain show damage in one or more instances. It is important to realize that no antipathy exists between the subcortical regions and the virus.

Evidence for Nerve Transmission

The inference that the nerve pathway is implicated in pathogenesis depends for its force on two requirements. First, the number of lesions in the brain must be small. Where the entire nervous system is a mass of inflamed tissue, no differential significance can be attached to the involvement of any particular part. Second, the nerve centers

implicated must be connected by simple and direct nerve páths. A
scheme whereby a circuitous nerve connection is imagined, involving
several intermediate stations and tracts of doubtful existence, is in-
genious but not convincing.   The force of the "nerve path transmis-
sion," theory is proportional to the directness of the connecting path-
way, and inversely proportional to the total number of lesions.

The best example of the importance of nerve connections in the
spread of the virus is offered by No. 8.   Here inoculation was into the
eye, and the lesions involve, in a quite selective manner, the entire
optic pathway up to the cortex.   Other unrelated lesions will be dis-
cussed below.   In No. 5, with virus injected into the pads, there is a
strikingly similar distribution of lesions.   In this latter case, the
virus carried by the blood attacked some part of the optic pathway,
and then spread out along the nerve paths of the visual system.

The utilization of existing nerve paths in the spread of virus within
the brain is shown by the olfactory connections.   Virus instilled into
the nose (No. 6) affected the olfactory bulbs, the pyriform lobes, and
entorhinal cortex, which are connected with the bulbs by the lateral
olfactory tract.   The other lesions will be discussed below.   In No.
4, a pad inoculation, the bulbs and pyriform cortex are affected in a
manner very similar to the previous case.   Similarly in No. 7, the
right olfactory bulb and the right anterior pyriform cortex both con-
tain lesions.   Here too the obvious link between the two involved
regions is the nerve pathway.

These examples suffice to show that in certain instances, where
there are relatively few lesions in the entire brain, anatomically
related nerve centers may be involved in a quite selective and striking
fashion.   There are two possibilities: either pure coincidence has oc-
curred, or the nerve connections have had some part in the spread
of the virus.   The latter choice alone seems plausible in the instances
cited.

Evidence for Spread by Direct Contiguity

When two contiguous portions of the nervous system are involved,
the relationship may be one of simultaneity and independence; or,
as in the preceding section, a spread from one region to the other by
means of nerve connections; or, third, direct extension from one area

to the other, as by passive transport of virus particles in the interstitial fluid.

It is very difficult to rule out possible nerve spread, for the short nerve connections of different regions of the brain are incredibly numerous. But in No. 3 a crucial example is presented, namely, the involvement of adjacent regions where nerve connections are known not to exist. The optic tract on the left is severely damaged, and the contiguous hypothalamus, medially, and amygdala, laterally, also show inflammatory changes. Neuronal relationship between the tract and these adjacent regions does not exist. The alternative of spread by direct extension appears overwhelmingly probable.

Many other probable examples exist in the foregoing protocols, but this mode of spread once established does not need elaboration.

Evidence for Blood Stream Transmission

The virus, once it has attained the central nervous system, may at times spread along the nerve processes or axones, that is, by nerve transmission; or it may spread by direct extension. But how does the virus reach the central nervous system following a subcutaneous inoculation?

In the present study the evidence for passage directly from the blood stream into the brain is the converse of that used to demonstrate nerve transmission. The latter evidence depends for its force on the involvement of successive nerve levels connected by known anatomical pathways. Conversely, the isolated involvement of a given center, all of whose known nerve connections are normal, is presumptive evidence that the nerve pathway is not involved. Blood stream transmission is the only reasonable alternative.

It is readily seen that in the brains previously described the majority of lesions occurred independently of nerve pathways. A few obvious examples will suffice. In Nos. 5 and 9, there are isolated lesions in various parts of the rhinencephalon, but the olfactory bulbs, where all incoming olfactory fibers end, are entirely normal. In Nos. 2 and 3, there are lesions in the acoustic centers of the midbrain and thalamus, yet the acoustic tubercles, where the peripheral nerves end, are normal. Brain 6 has a lesion in the striate (visual) area, but the corresponding lateral geniculate body is intact. Similarly

No. 3 has a well marked focus in the left limbic (infraradiar) region, but the anterior nucleus of the thalamus, which is the afferent subcortical center, is unaffected. Brain 7 shows a massive focus of destruction in the postcentral (sensory) cortex, yet the entire thalamus is normal. Several brains show small foci in the cerebellar cortex, but the cerebellar connections are normal.

The frequency of discrete small lesions in the neocortex, together with the intact state of the subcortical centers, is readily apparent. The virus injected into the periphery appeared in the highest level of the nervous system without any evidence of having passed through the appropriate lower centers. To travel along nerve paths from the periphery to the cortex, certain primary and intermediate nuclei must be traversed. Severe lesions in the higher centers, with perfectly normal lower centers, would seem to exclude the utilization of nerve paths. The only reasonable alternative is that the virus attacked the cortex directly through the blood vessel wall.

The Rôle of the Spinal Fluid

Were the cerebrospinal fluid of significance in the spread of the virus through the nervous system, an entirely different histological picture would be called forth. It is known that this virus is capable of attacking the leptomeninges. Consequently a primary meningoencephalitis, with fairly uniform distribution of lesions at the margins of the spinal fluid pathway, would be expected. This has not been observed. Instead, sharply circumscribed focal lesions appear, not only on the surface of the brain, but also deep in the hemisphere and brain stem. There appears no way to harmonize these data with any possible rôle of the spinal fluid in the pathogenesis of this disease.

DISCUSSION

The importance of the blood in the pathogenesis of this disease is beyond question. The virus is known to circulate in high titer. We see further that after a constant mode of inoculation, as by pad injection, or intraocular inoculation, the distribution of lesions is inordinately varied. The blood stream is the only agent which could account for such vagaries.

This is of especial interest in relation to intraocular or intranasal

inoculation. Out of three cases of intraocular injection presented, in only one was there clearly an involvement of the optic system. Even though the virus may utilize nerve connections from the eye there is still an escape into the blood. Virus once in the blood acts essentially at random, regardless of the site of inoculation. In such instances as Nos. 6 and 8, the primary spread is related to the site of inoculation (nose and eye, respectively). But there are numerous totally unrelated lesions. These latter are attributable to secondary spread by way of the blood stream, and not by way of hypothetical nerve paths as yet undiscovered.

That the spread of viruses may be determined by existing nerve paths is an inference. This inference has been invoked by many authors in the study of different neurotropic viruses (8). In the present study of the guinea pig, the involvement of related nerve centers is sometimes extremely striking. For example, Nos. 4, 5, 6, and 8 show an involvement of successive neurones of a given system (olfactory or visual), with relatively little damage to the remainder of the brain. The probability is overwhelmingly high that this selective involvement was not merely due to chance: in some way the anatomical connections influenced the spread of the virus.

In certain other instances the probability of nerve spread is less strong—much less so. For example, in No. 3 there are a single lesion in the left olfactory bulb and single lesions in the posterior pyriform cortex, both right and left. There may be some causal connection between these three lesions; that is, the known nerve connections might be invoked as a connecting link. But there might, in this case, be a relationship merely of coincidence. If we compare the olfactory regions of Nos. 1 or 3 with those of Nos. 4 or 6, we see a marked difference. In the two latter instances, nerve spread seems definite; in the two former, it is problematic. Coincidence is equally or even more likely, especially in view of the spotty distribution of lesions elsewhere in the nervous system.

On the other hand, in this series the majority of lesions in the brain, especially in the neocortex, do not bear any relationship to each other according to known anatomical connections. In such instances a nerve spread of virus seems overwhelmingly improbable. The alternative of direct spread from the blood stream is the only satisfactory explanation.

There is only one criterion of nerve spread, namely, the topographical relationship of affected areas. Certain authors (4, 5) have held that the perivascular nature of some lesions is an indication that the virus passed through the blood vessels. In the present series of cases, where several hundred lesions were studied, this distinction is found not to obtain. No histological difference could be observed between regions where the virus was presumably blood borne, as compared with regions where nerve transmission presumably occurred. Only the anatomical relations of the affected regions, compared with each other and with the remainder of the nervous system, furnish a ground for distinction.

It is thus seen that the virus of equine encephalomyelitis is bipotential. It enters the brain from the blood stream through the blood vessel wall. Once in the nervous system, it may or may not spread along preexisting nerve paths. No explanation can at present be offered why in one instance it does and in another does not.

A question which must be kept open is that of possible deposition of virus from the blood on the olfactory mucosa, with subsequent spread along the olfactory connections (Hurst (2), Sabin and Olitsky (4)). The mere presence of lesions somewhere in the olfactory brain is obviously not sufficient to establish this theory. However, a marked involvement of the olfactory bulbs is entirely consistent with the hypothesis. In the present series of cases, No. 4 might be interpreted in this sense if one so desired. In the present state of our knowledge, this theory must be held under reserved judgment. There can be no doubt that in reference to certain neurotropic viruses the nose and the olfactory pathway stand in a different category from most other sense modalities and other parts of the body. The reason is as yet obscure, and further studies in anatomy as well as pathology are necessary.

The findings in the guinea pig may be compared with the results obtained by Sabin and Olitsky (4) in young mice, which alone were found susceptible to peripheral injection. According to these authors, the virus may occasionally invade the central nervous system along the local peripheral nerves. More often, they believe, virus transported by the blood invades by means of the olfactory or auditory pathways, or possibly along the seventh nerve. "No evidence was found of a direct passage of virus across the blood vessels of the brain" (9).

In the guinea pig, occasional animals show primary involvement of

the olfactory pathway, and there is no way to rule out a prior deposition of virus on olfactory mucosa. But in no other case was there evidence of spread *via* the peripheral nerves; that is, in no other case were the primary receptive nuclei involved. The cases of spread along the optic nerve are not exceptions, since the optic nerve is not a peripheral nerve at all. With the possible exception of the olfactory nerve, the virus seems to have attacked the central nervous system directly, without the intermediation of the peripheral nerves. This attack is considered to take place by passage across the blood vessels of the brain.

SUMMARY

After inoculation with equine encephalomyelitis virus by various routes, guinea pigs were sacrificed at early stages, before symptoms were apparent. The brains were studied histologically, with serial sections; all lesions were noted, and subjected to topographical analysis. Nine cases are presented in detail.

With any given mode of inoculation the distribution of lesions varied very widely from one instance to another. In some cases, affected regions bore a striking and definite anatomical relationship to each other. These distributions can be explained only by the assumption that the anatomical pathways played some rôle in the spread of the virus. In other instances lesions were present in areas, the anatomical connections of which were entirely normal. Attention is called to the frequency of lesions in the neocortex, with intact subcortical centers. Such distribution is held to render nerve spread extremely improbable. The only satisfactory explanation of such random distributions is by direct passage of virus from the blood stream into the brain tissue. There is no histological difference between lesions which result from blood spread and those resulting from nerve spread.

CONCLUSIONS

In the guinea pig, the virus of Eastern equine encephalomyelitis, injected peripherally, invades the blood stream and passes directly from the blood stream into the brain. This seems to be the principal, though not necessarily the exclusive, mode of pathogenesis. Once in

the nervous system the further spread of the virus may occasionally be determined by anatomical connections.

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EXPLANATION OF PLATE 40

FIGS. 1 and 2.   Two typical lesions, from guinea pig 1.   Thionin.   Fig. 1, × 43.   Fig. 2, × 65.
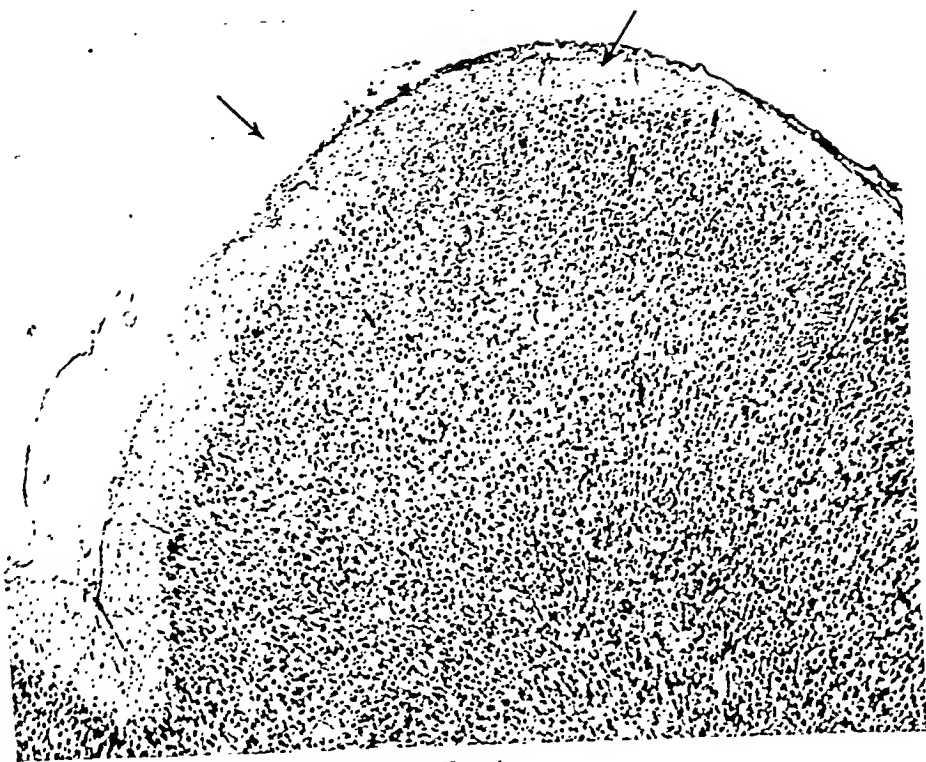
FIG. 1
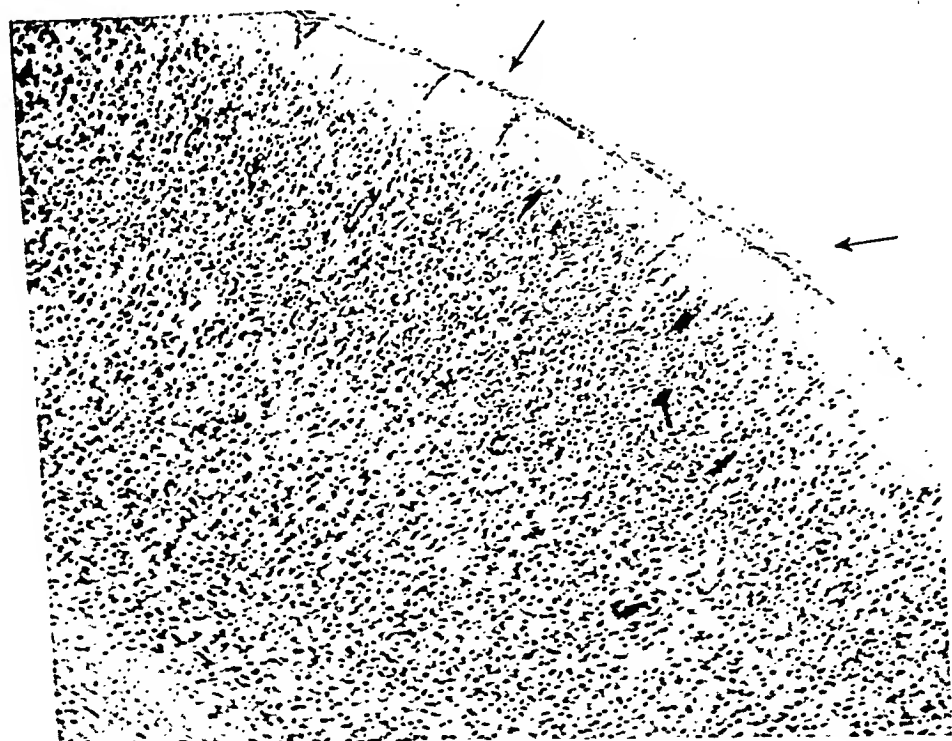
FIG. 2

STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

III. INTRAOCULAR INFECTION WITH FIXED VIRUS IN THE GUINEA PIG

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PLATE 41

In the preceding paper (1) it was shown that a recently isolated strain of equine encephalomyelitis virus, when injected into the eye, sometimes spread along the optic pathways. This type of spread was, however, inconstant. But with a fixed strain of virus (2, 3) an entirely constant behavior was observed which was capable of systematic study.

For the study of virus activity within the central nervous system, inoculation into the eyeball presents certain great advantages due to the anatomical peculiarities of this organ, which make it the most readily accessible portion of the central nervous system.

Anatomy.—In general, the peripheral and central nervous systems differ fundamentally in the type of accompanying stroma. Within the brain or spinal cord there are glial cells which form the supportive tissue, but no true connective tissue stroma. Small quantities of connective tissue accompany the blood vessels but are shut off from the true nerve parenchyma by the pia-glial membrane. On the other hand, in the cranial or spinal nerves, including the sensory ganglia, there is a rich stroma of fibroblasts and associated cells, collagen, and reticulin, with a true lymphatic system. The junction between the central and the peripheral portions of the craniospinal nerves has recently been well described by Tarlov (4). The importance of this stroma in relation to certain problems of the so called hemato-encephalic barrier has been emphasized in recent publications (5, 6).

The optic nerve is not a peripheral nerve at all, in the sense applied to the vagus, hypoglossal, or any of the spinal nerves, for example. Instead, the optic nerve is embryologically and anatomically a tract of the brain substance proper, having all the characteristics of the central nervous system.

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It is well known, of course, that the optic pathway in the lower vertebrates is chiefly though not entirely crossed, the decussation taking place in the optic chiasm. The principal connections of the right eye, for example, are with the left lateral geniculate body, and pretectal nucleus of the thalamus, and the left superior colliculus. The converse obtains with the left eye. From the lateral geniculate body a projection tract goes to the striate cortex of the same side.

Method.—In the experiments herein reported, a 27 gauge needle was inserted from the inner canthus of the eye directly through the sclera into the posterior chamber of the eye. Passage through the anterior chamber was avoided. The injection mass (0.03 to 0.05 cc.) was placed in contact with the ganglion cells of the retina and the overlying fibers of the optic nerve.

The virus was thus placed in the vitreous humor, in contact with the cells and fibers of the central nervous system with essentially no trauma to the latter.

Strains of Virus.—For the most part a strain of Eastern equine encephalomyelitis virus that had been modified by serial intracerebral passage in pigeons was employed. This strain was developed by Traub and TenBroeck (2), and has been further studied in detail by Traub (3). In the present experiments the 112th to 117th pigeon passages were utilized. In addition certain comparative experiments were carried out with an unmodified strain of the Eastern virus, isolated from a horse in 1937, of which the 2nd to 5th intracerebral guinea pig passages were utilized. All titer figures refer to tenfold dilutions of a 10 per cent suspension of pigeon (or guinea pig) brain, which is designated as 10^0 dilution.

Sensitivity of the Intraocular Route

To determine the relative sensitivity of the intraocular as compared with the direct intracerebral route, comparative titrations were performed using the same amount of inoculum for each route. Both the fixed strain and the unmodified strain were used. The results of two such titrations are given in Table I. In some experiments fatalities have resulted from a 10^{-3} dilution of the fixed strain, and as high as 10^{-6} of the unmodified strain, injected intraocularly.

With the fixed strain there is a definite difference between the intraocular and the sub- or intracutaneous routes. Traub (3) and TenBroeck and Traub (2) have already found that guinea pigs are quite insusceptible to subcutaneous inoculation, and the present work fully confirms their results. With the dosage used (0.05 cc.) the undiluted virus suspension regularly failed to produce encephalitis following an intracutaneous injection, although the intracerebral titer was 10^{-6} or 10^{-7} (with the same dosage).

With the unmodified virus, on the other hand, repeated experi-

ments with the same quantity of inoculum did not reveal any significant difference between the intraocular and intracutaneous routes of administration.

With the fixed virus, it is clear that the susceptibility of the guinea pig to intraocular inoculation is midway between the intra- or subcutaneous and the intracerebral routes. This difference does not obtain with the unmodified virus.

TABLE I

Titration of Intraocular and Intracerebral Inoculations in Guinea Pigs

Dilution	Fixed strain (pigeon passage)		Unmodified strain	
	Intraocular inoculation	Intracerebral inoculation	Intraocular inoculation	Intracerebral inoculation
10^{-8}	N.T.	N.T.	0, 0	0, 0
10^{-7}	"	2, 0, 0	0, 0, 0	5, 9, 0
10^{-6}	"	3, 0, 0	0, 0, 0	3, 4, 6
10^{-5}	"	2, 3, 3	4, 6, 0	3, 3, 4
10^{-4}	0, 0, 0	2, 2	N.T.	N.T.
10^{-3}	0, 0, 0	N.T.	"	"
10^{-2}	3, 4, 0	"	"	"
10^{-1}	3, 3, 5	"	"	"

N.T. = not tested.

0 = guinea pig survived.

2, 3, 4 = guinea pig died in 2, 3, or 4 days after the inoculation.

Action of the Virus within the Eyeball

When a 10^{-1} dilution of the fixed virus is injected into the eye, the animal invariably dies, usually from 72 to 96 hours after the injection. With this mode of inoculation it is easy to determine the minimum length of time the virus must act in order to produce a fatal infection. In a series of experiments the injected eyeball was surgically removed under deep ether anesthesia at different intervals following the inoculation. The eyeball can easily be removed intact, hemorrhage is negligible, and the animal always makes an uneventful recovery. A small quantity of pus may occasionally form in the eye socket but never causes symptoms of any sort.

The results of three such extirpation experiments following the injection of fixed virus are given in Table II. It is seen that when the

eye was removed in less than 6 hours, only 2 out of 17 animals died. When removal was performed 10 to 13 hours after inoculation, 8 out of 14 succumbed.   All controls died.   The period of 10 to 13 hours seems to be the average minimum time in which the virus must act within the eyeball in order to produce a fatal infection.

TABLE II

Guinea Pig Survival after Removal of Eyeball at Different Intervals after Injection with·Fixed Strain of Virus

Experiment No.	Inoculated eye removed after					Controls
	1 hr.	3 hrs.	6 hrs.	10 hrs.	13 hrs.	
1	5, 0	0, 0*	0*, 0*	3, 3	N.T.	3, 3
2	0, 0*	0, 0, 0	4, 0, 0	3, 3, 0	3, 0	3, 6
3	N.T.	N.T.	0, 0, 0	3, 0, 0*	3, 3, 0, 0	3, 3

*In subsequent tests of immunity to intracerebral inoculation of 3×10^3 to 10^4 minimal lethal doses, the starred animals survived.   Those not starred were not immune.

Other abbreviations as in Table I.

TABLE II*a*

Identical Procedure with Unmodified Strain

Experiment No.	Eye removed after			Control
	½ hr.	1 hr.	3 hrs.	
1	6, 8	5, 0	4, 8	5, 0
2	5, 0	4, 5	5, 6	4, 0*
3	4, 0	5, 6	0, 0	4, 5, 5

*This animal showed marked signs of encephalitis, but recovered.   Later histological examination showed healing encephalitis.

Survivors were not tested for immunity.

In the foregoing experiments the survivors were tested for immunity by the intracerebral injection of 0.1 cc. of a 10^{-3} dilution of virus, whose virulence by intracerebral tests in mice was constantly 10^{-6} or 10^{-7}.   The animals that survived the immunity test are designated by a star in Table II.   There is no constancy in the induction of immunity.   One animal whose eye was removed 1 hour after inoculation was immune.   Other animals, with removal after 13 hours, were not immune.   The reasons for this variability are not clear.

Extirpation experiments were also carried out with the unmodified strain, as shown in Table II *a*. The fairly definite time interval required with the fixed strain is not necessary for the fresh virus. This property is undoubtedly correlated with the relative abilities of the two strains to invade the blood stream. The unmodified strain is readily found in the blood stream, but the fixed strain is recovered only rarely, or not at all (3).

Course of the Virus after Inoculation into the Eye

It was desirable to try to trace the course of the virus by examining different portions of the brain for virus content at different intervals of time after inoculation.

For this purpose 6 animals were inoculated into the right eye and sacrificed at different periods of time. The brains were removed aseptically and subdivided in accordance with the known optic pathways. The method of sectioning the brain was as follows: The cerebellum, medulla, and inferior colliculi were first cut off and discarded. With the brain ventral surface upward, gentle traction was applied to the optic chiasm, resulting in separation of the optic tracts from the underlying tissue. The chiasm and tracts were then cut away. The brain was turned dorsal side upward, and with small sharp scissors the lateral ventricle was entered from the medial surface of the hemisphere. The posterior neocortex of one side was then cut away, following the line of the rhinal fissure ventrally. The portion so removed included the entire area striata, and portions of the temporal, parietal, and occipital areas. It is impossible to differentiate these areas macroscopically. The procedure was repeated for the opposite side. Then the corpus callosum was cut through, and the two hippocampi and the hippocampal commissure gently peeled forward, exposing the thalamus and superior colliculi. The thalamus was separated from the hemispheres by incisions along the striae terminales. The thalamus and midbrain were then divided in the midline, and each half used separately. The entire olfactory portion of the brain, together with the basal ganglia and anterior neocortex, was left and utilized as a single portion. Throughout the dissection special care was taken to avoid contamination of one part by another.

This method of division gave 6 portions of the brain to be tested: the optic chiasm and tracts; the right thalamus plus superior colliculus; the left thalamus plus superior colliculus; the right and the left posterior neocortex; and the remainder of the hemispheres. The chiasm was ground up in 0.5 cc. of saline; the other portions were also ground in saline in approximately 10 per cent dilution. After light centrifugation, the supernatant fluid was injected intracerebrally into 3 to 4 week old mice, the dose being 0.05 cc.

The optic pathway was thus divided into three successive neurones: the optic chiasm and tracts, constituted by the axones of the ganglion cells of the retina; the lateral geniculate body and superior colliculus, where the optic tract terminates; and the visual cortex, which receives fibers from the lateral geniculate body. The thalamus and neocortex were tested bilaterally.

The results of this experiment are recorded in Table III. A minute trace of virus was detected in the contralateral geniculate body (and midbrain) 13 hours after the injection, although the optic chiasm did not show virus. At 24 hours there was abundant virus in the optic chiasm and the contralateral geniculate body and midbrain, but nowhere else. At 36 hours virus was present not only in these

TABLE III

Spread of Fixed Virus after Inoculation into the Right Eye.
Virus Content of Brain Portions, Tested by Intracerebral Inoculation into Mice

Guinea pig	Time of sacrifice	Optic chiasm	Left geniculate and midbrain	Left occipital cortex	Right geniculate and midbrain	Right occipital cortex	Remainder of brain
	hrs.						
1	10	0, 0, x	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
2	13	0, 0, 0	8, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3	24	3, 3, 4	2, 3, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
4	36	2, 2, 3	2, 2, 3	2, 2, 0	0, 0, x	0, 0, 0	0, 0, 0
5	48	2, 2, 2	2, 2, 2	2, 2, 3	3, 4, 5	2, 4, 0	2, 4, 4

x = death occurred within a few hours of the inoculation, and was not due to virus action.

two regions, but also in the visual cortex of the contralateral side; other portions of the brain were still virus-free. At 48 hours, however, virus was widely disseminated throughout the entire brain.

The results of this experiment are entirely consistent with the view that the virus, inoculated into the eye, spread along the optic pathway to the thalamic centers (and midbrain), and from the thalamus progressed by the projection path to the cortex. These are the regions which first contain virus. The contralateral optic pathway was predominantly affected, since in the guinea pig the great majority of the optic fibers decussate.

To study further the time relationships involved, the foregoing

experiment was repeated, but only the optic chiasm and the left thalamus and midbrain were tested for virus content.   Again inoculation was into the right eye.   The results are given in Table IV. Again minute quantities of virus were detectable as early as 12 and 17 hours after inoculation, but significant quantities were first present at 20 to 24 hours.   An important feature to be noted is the pres-

TABLE IV

Relation between Virus Content of Optic Chiasm and Tracts and of the Secondary Optic Centers, Following Inoculation into the Right Eye. Tested by Intracerebral Inoculation into Mice

Time	Experiment No.	Guinea pig	Optic chiasm	Left geniculate and midbrain
hrs.				
12	2	1	0, 0, 0	12, 0, 0
14	1	2	0, 0, 0	0, 0, 0
	2	3	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0
17	1	5	8, 0, 0	0, 0, 0
	2	6	0, 0, 0	0, 0, 0
		7	0, 0, 0	0, 0, 0
20	1	8	0, 0, 0	0, 0, 0
	2	9	2, 3, 0	0, 0, 0
		10	0, 0, 0	0, 0, 0
24	1	11	2, 2, x	2, 3, 4
	2	12	0, 0, 0	0, 0, 0
		13	2, 2, 0	0, 0, x

ence of virus in fairly large quantities in the optic chiasm and tracts before virus was apparent in the thalamus or midbrain (guinea pigs 9 and 13).

Pathology

The histopathology of the brain after infection with the fixed strain of virus has been found by Traub (3) to be substantially the same as with the unmodified virus.   The first paper of this series (7) has

described in considerable detail the pathology of the natural virus disease in the guinea pig.   Further study with the fixed strain reveals certain differences.   After intracerebral inoculation the inflammatory reaction is vastly more pronounced, and the hippocampal degeneration is much more slight than after similar inoculation with the natural virus.   The intensity of the inflammatory reaction is especially surprising in view of the considerably shorter duration of the disease (generally 2 to 3 days rather than 3 to 5).

In the present study, however, there are three principal points of interest in the pathology: the reaction occurring within the eye, the nature of the first lesions to appear within the brain, and the distribution of these lesions.

Intraocular Pathology.—Sections of eyes removed at different intervals, cut serially at 7 microns with short ribbons mounted every 50 sections, showed the development of the process.   At about 13 hours there is a very slight reaction consisting of serum exudation, and a few mononuclear cells within the eyeball. In the connective tissue coats of the eye there may be numerous polymorphonuclear leucocytes.   Within the eyeball, however, in uncomplicated cases, there is surprisingly little pathological change, even when the eye is removed at the death of the animal.   A few leucocytes are present in the vitreous humor, and occasionally scattered leucocytes penetrate the outer layers of the retina.   But there is no necrosis of tissue, and none of the focal reaction that is so prominent in the brain substance elsewhere following intraocular injection.

Sometimes there is a pronounced accumulation of polymorphonuclear leucocytes within the eyeball, and then the invasion of the surface of the retina by leucocytes is more intense, and necrotic ganglion cells may rarely be seen.   This, however, is an entirely non-specific reaction.   This histological picture has been duplicated by injection of normal brain emulsion diluted to the same degree as the virus suspensions used.   Furthermore, when large numbers of leucocytes are present, intracellular bacteria may usually be seen in the exudate.   Eyes that are sterile by bacteriologic culture always show an insignificant reaction to the virus (Figs. 1 and 2).

It may be mentioned that the slight degree of intraocular contamination sometimes becoming noticeable 24 or more hours after inoculation, has never had the slightest effect on the action of the virus.

Nature of the Cerebral Lesions.—In contrast to the lack of reaction in the eye, the histological picture in the higher optic centers is identical with that already described for the unmodified, natural virus.   In 9 animals, sacrificed at intervals of from 24 to 52 hours, the entire thalamus and superior colliculus was sectioned serially at 10 microns, and every 15th section examined.   In 5 other cases, from

48 to 65 hours, the entire brain was sectioned. Hematoxylin and eosin, phloxin and methylene blue, and thionin were the stains used.

The earliest change, first found at 24 hours, is a mild perivascular reaction, consisting of mobilized glial cells and mononuclear leucocytes (Fig. 3). This speedily develops into the typical inflammatory focus already described for the natural strain. As early as 36 hours after inoculation the typical focus may be very intense (Fig. 4). The earliest lesions are always found in the contralateral geniculate body or superior colliculus, followed 12 to 15 hours later by similar lesions in the same centers of the side of the inoculation. The series of cases studied, therefore, has double opportunities for observations of early lesions, first on the contralateral, then on the ipsilateral side.

Since the virus appears to spread along the nerve paths, it might be thought that a terminal nerve center might show neuronal necrosis as the first sign of disease. This is very definitely not the case. A closely graded series of cases supports the finding already published (7), that a vascular and interstitial reaction is the first sign of disturbance. These data further support the conclusion previously expressed (1), that there is no difference between lesions caused by blood-borne and those caused by nerve-borne virus.

The later lesions present no new features beyond what has already been described (7).

Distribution of Lesions.—The relatively crude method of gross dissection of the brain and testing of each portion for virus content, indicated that a spread of virus along the optic pathway was likely. The method of topographical analysis of the lesions (1) entirely supports the previous evidence. As early as 24 hours after right-sided injection there may be definite histopathology in the left superior colliculus or lateral geniculate body, rapidly growing more severe. The first lesions are sharply focal and rather rare, but they rapidly become more numerous and more intense until a maximum is reached at about 48 hours. Early lesions on the right side may be detected at 38 hours. At 48 hours the process has extended fairly widely into adjacent thalamic nuclei, so that large portions of this subcortical center are affected. The optic chiasm and optic tracts first show lesions at 36 to 38 hours, which is significantly longer than the time required to produce injury at the terminus of these tracts. The basal meninges begin to show inflammatory changes at about the same time as the optic tracts.

Pathologic changes in the left visual cortex are present to a very slight degree at 48 hours, but rapidly become more severe. At 53 to 55 hours there may be slight changes in the right posterior neocortex, but the temporal and occipital areas are more involved than the striate area.

At 48 to 53 hours there are numerous demonstrable lesions in areas which are functionally independent of the visual pathway. Such lesions may be present in various portions of the olfactory pathway, especially the tuberculum olfactorium and the amygdala, but also the septum; in the basal ganglia; in scattered unrelated areas of the anterior neocortex; in the hypothalamus, midbrain, and medulla.

DISCUSSION

Susceptibility to intraocular inoculation of fixed virus, as has been pointed out, lies intermediate between the intracerebral and the sub- or intracutaneous routes. The latter is fatal only with massive doses. The intracerebral route is always fatal in high dilutions. The intraocular route is fatal only in low dilutions (10^{-2} or 10^{-3}). The mechanisms involved appear to be somewhat different in the different methods.

The simplest explanation would seem to be as follows. Virus introduced into the eye infects the superficial ganglion cells of the retina. Once the cell body is infected, the entire neurone speedily becomes involved. That is, the virus then infects the cell processes making up the optic nerve. In the case of intraocular injection this takes place entirely within the central nervous system.

Such a spread of virus is quite different from that following a peripheral inoculation; with virus injected, say, into the thigh, any possible passage up the local nerve must infect the terminus of the nerve first, with subsequent passage toward the cell body. The entire metabolism of the neurone is controlled by the cell body. Passage of virus away from the cell body (centrifugal spread) seems to be in a different category from centripetal spread, at least, so far as this virus is concerned. The mechanism by which the virus, once it has infected the cell body, can "travel" along the axone, still remains completely unexplained. The multiplication of virus occurs with ex-

traordinary rapidity, and over a significant distance. Any attempted explanation at present would be sheer speculation.

The time interval of 10 to 13 hours required for virus to act within the eye is capable of different interpretations. This interval may represent the time required for the virus to work its way into the cell and begin its action. Or, it may include the time actually necessary for the virus to pass the length of the optic nerve and tract, and reach the geniculate body. There are no grounds as yet for a definite decision.

In Table II, Experiment 1, one guinea pig died although the eye was removed after an hour. This instance is similar to the behavior of the natural virus (Table II a) and may be an indication that in rare instances the fixed virus may act like the unmodified strain.

Some of the other animals whose eyes were removed and which yet survived, were immune to subsequent intracerebral inoculation. Undoubtedly a small amount of the virus escaped into the blood stream, not enough to produce fatal infection, but sufficient to immunize.

There are certain points of interest in the histological studies. Within the eye there is a negligible reaction although there the virus acts first and longest. On the other hand, elsewhere in the central nervous system the histological reaction is typical and in complete accordance with the descriptions previously given. This behavior of the eye appears strictly comparable to the behavior of brain tissue toward intracerebral inoculation. In the latter case, the site of injection of the virus shows merely a non-specific reaction to injury, although elsewhere in the brain there is well marked encephalitis. Furthermore, in another connection, guinea pigs have been infected by injection of virus into the cistern, so that the inoculum directly entered the cerebrospinal fluid. Here too the contact of virus and brain tissue caused only a mild and completely insignificant reaction. Characteristic pathology, found within the parenchyma, was not present at the surface of the brain in contact with the virus.

Experimental juxtaposition of virus and nerve tissue does not call forth the same reaction produced by the virus after its natural mode of spread. An analogy may be drawn with the data of vital staining

where trypan blue, for example, placed in direct contact with the brain, acts entirely differently from the same dye brought to the brain by the blood stream. These facts, and their relation to the "blood-brain barrier," are elsewhere discussed (6).

SUMMARY

The behavior of a fixed strain of Eastern equine encephalomyelitis virus was studied in guinea pigs after intraocular inoculation. Such inoculation concerns the central and not the peripheral nervous system.

The susceptibility to intraocular injection lies midway between the highly virulent intracerebral and the quite avirulent peripheral routes. The virus must act for 10 to 13 hours in order to induce a fatal infection. Removal of the inoculated eyeball before this interval almost always prevents fatality although it may allow immunity to develop. The virus, at suitable intervals after injection into the eye, may be recovered from successive and appropriate optic centers before it is demonstrable in non-optic portions. Approximately 24 hours are required for the virus to reach a significant concentration in the contralateral geniculate body, 36 hours in the contralateral visual cortex. Significant amounts of virus may be present in the optic chiasm and tract prior to involvement of the higher centers.

Virus placed in contact with the retina produces an insignificant, essentially non-specific reaction comparable to that produced at the site of direct intracerebral inoculation. In the retina there is no ganglion cell necrosis unless there is a complicating intraocular infection. In the cerebral visual centers the first reaction is inflammatory and interstitial, and may appear in the lateral geniculate body as early as 24 hours after injection. Neuronal necrosis is not the primary action of the virus on the nervous system in these experiments. The distribution of lesions in the brain is in excellent agreement with the method of direct testing for virus content, and is far more accurate than the latter.

The virus in its primary distribution through the nervous system follows the nerve pathways of the optic system. This occurs within the central nervous system, where presumably there is first an in-

volvement of the nerve cell body and then a spread along the cell process or axone.

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EXPLANATION OF PLATE 41

FIGS. 1 and 2. Retina of injected eye of guinea pig. Eye removed at death, 66 hours after inoculation. In Fig. 1 are a few leucocytes on the surface of the retina; in Fig. 2, within its substance. The ganglion cells, as well as other elements, are intact. Phloxin-methylene blue. × 152.

FIG. 3. Lateral geniculate body, contralateral to the injected eye. 24 hours after inoculation. The area indicated by the arrows shows a sparse infiltration with leucocytes and blood mononuclears, and some glial proliferation, all in relation to the blood vessel. There is no ganglion cell necrosis. Hematoxylin-eosin. × 152.

FIG. 4. Superior colliculus, contralateral to the injected eye. 36 hours after inoculation. There is a very intense, circumscribed focus consisting chiefly of polymorphonuclear leucocytes. Apart from the inflammatory area, there is no neuronal necrosis. Hematoxylin-eosin. × 56.

PLATE 41

(King: Eastern equine encephalomyelitis. III)
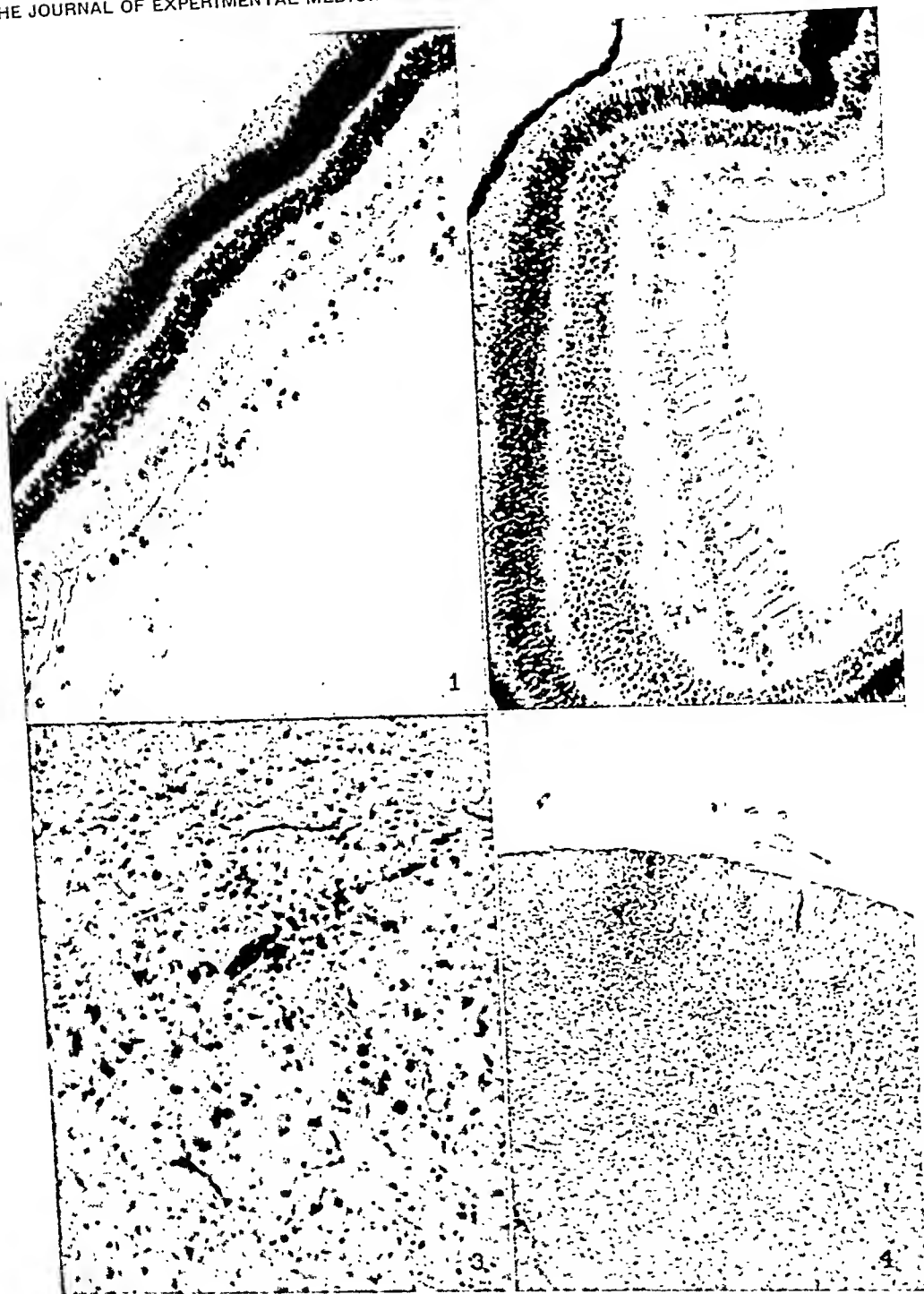
ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES. III

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For the purpose of studying the serological behavior of synthetic substances of somewhat complex structure, which at the same time bear a relation to natural antigens, we have proceeded to investigate immune sera to azoproteins made from longer peptide chains than before (1, 2), that is, pentapeptides; the latter again were built up from glycine and d,l-leucine. Since in previous studies the terminal amino acid, with free carboxyl group, dominated to a considerable extent in the serological reactivity, peptide amides have now been included in order to eliminate the prominent influence of acid groups and to get information on the rôle played by other parts of the molecule in the reactions with immune bodies. The peptides used for the preparation of azo antigens were tetraglycyl-glycine, tetraglycyl-leucine, d,l-diglycyl-leucyl-glycyl-glycine and trileucyl-glycyl-glycine.[1]

EXPERIMENTAL

The preparation of a number of the substances used has been described previously (1, 2). With some of these compounds, namely the first four mentioned below, more convenient methods have now been utilized. Melting points were not corrected and mostly no attempt was made to recrystallize to constant melting point.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine.—7.4 gm. of *p*-nitrobenzoyl-glycyl-glycine hydrazide (see below) were dissolved in 15 cc. 5/n HCl, 100 cc. of 50 per cent acetic acid, and 150 cc. of water, and were converted into the azide at 0-5°C. by slow addition of a solution of 2.6 gm. of sodium nitrite in 10 cc. of water. After ½ hour at 0-5°C. the azide was filtered off and washed with cold water until

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[1] These compounds are hereafter designated respectively G₅, G₄L, G₂LG₂, L₃G₂, and the amides by the symbol Am. The preparation trileucyl-glycyl-glycine may well be a mixture of steric isomers although the nitrobenzoyl derivative was crystalline. (For other abbreviations see references 1, 2.)

free from acid.   The azide was dissolved in 250 cc. of cold 85 per cent alcohol and the solution was added to a neutral solution of 7.4 gm. of diglycyl-d,l-leucine in 300 cc. of water.   The mixture was stirred and 40 cc. of N/NaOH were added in small portions over a period of 1 hour to keep the solution slightly alkaline to litmus.   After 2 more hours the solution was made weakly acid to litmus and concentrated *in vacuo* at 40° to a volume of 150 cc.   If the solution became alkaline to litmus during the distillation, it was neutralized with HCl.   After removal of a small amount of alkali insoluble material by filtration, the solution was made acid to Congo red.   The sticky precipitate crystallized upon rubbing (needles). Yield 7.5 gm.   It was recrystallized from water and again from 60 volumes of 50 per cent alcohol.   Bushels of needles, m.p. unsharp with decomposition at 240°C. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.49.

p-Aminobenzoyl-Tetraglycyl-d,l-Leucine.—The nitro compound was dissolved in 50 volumes of 75 per cent alcohol and reduced by means of palladium black and hydrogen at atmospheric pressure.   The solution was evaporated to dryness *in vacuo* and the amino compound was recrystallized from 50 per cent alcohol. Microcrystalline.   Yield 65 per cent.   M.p. 165–166°C.   Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.50.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—This substance was prepared by the above method using 7.4 gm. of *p*-nitrobenzoyl-glycyl-glycine hydrazide and 7.4 gm. of d,l-leucyl-glycyl-glycine.   After completion of the reaction and concentration *in vacuo* the solution was made acid to Congo red by addition of HCl and kept overnight in the ice box.   The crystalline precipitate (needles) was filtered and recrystallized from 100 cc. of water.   Yield 6 gm.   M.p. 144–145°C.   Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.61.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine.—The nitro compound was reduced by means of palladium and hydrogen as described, the alcoholic solution was evaporated to dryness *in vacuo*, the amino compound was dissolved in a small amount of methyl alcohol and precipitated by addition of dry ether.   The precipitate became granular upon rubbing with more dry ether.   Yield 90 per cent. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.41.

Esters and Amides of Nitrobenzoyl Amino Acids and Nitrobenzoyl Peptides.— The nitrobenzoyl compounds (1,2) were converted into methyl esters by either dissolving or suspending the finely ground substance in 5 parts of absolute methyl alcohol and adding in portions an ether solution of diazomethane at 0–5°C., using a slight excess.   The mixtures were allowed to stand at room temperature and after removal of the solvents the substances were further purified as described below.   The esters were changed into amides by dissolving them in 100 parts (or more) of hot absolute methyl alcohol and saturating the solutions with dry ammonia gas at 0–5°C.   The solutions were kept in a closed flask at room temperature for 48 hours and were then evaporated to dryness *in vacuo*; the last traces of ammonia were removed by again adding dry methyl alcohol and evaporating to dryness.   The amides were further purified by recrystallization.

Reduction of Nitrobenzoyl Compounds.—The aminobenzoyl amino acid amides and aminobenzoyl peptide amides were prepared by suspending the finely ground nitro compounds in 50 volumes of 75 per cent ethyl alcohol (some were completely and others only partly dissolved) and reducing by means of palladium black and hydrogen at atmospheric pressure. After complete reduction no undissolved substance was left. The solutions were filtered and evaporated to dryness *in vacuo.* The amino compounds were further purified as described below.

p-Nitrobenzoyl-Glycine Ethyl Ester.—This was prepared by nitrobenzoylation of glycine ethyl ester by the method used previously for *p*-nitrobenzoyl-tyrosine ethyl ester (3). The chloroform solution of the nitrobenzoyl compound was evaporated to dryness *in vacuo* and the substance was freed from nitrobenzoic acid by dissolving in a small amount of hot chloroform and precipitating with 5 volumes of ether. It was recrystallized from 5 parts of absolute ethyl alcohol. Narrow platelets. Yield 16 gm. from 14 gm. of glycine ethyl ester hydrochloride. M.p. 141–143°C. Analysis: Calculated for $C_{11}H_{12}O_5N_2$: N 11.11, found 11.05.

p-Nitrobenzoyl-Glycine Amide.—Recrystallized from 30 parts of 80 per cent ethyl alcohol. Platelets. M.p. 239–240°C. Yield 80 per cent. Analysis: Calculated for $C_9H_9O_4N_3$: N 18.83, found 18.66.

p-Aminobenzoyl-Glycine Amide.—Recrystallized from 80 parts absolute methyl alcohol. Platelets. M.p. 225–226°C. Yield 70 per cent. Analysis: Calculated for $C_9H_{11}O_2N_3$: N 21.76, found 21.82.

p-Nitrobenzoyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Needles. M.p. 83–84°C. Yield 72 per cent. Analysis: Calculated for $C_{14}H_{18}O_5N_2$: N 9.52, found 9.48.

p-Nitrobenzoyl-d,l-Leucine Amide.—Recrystallized from 25 parts of 80 per cent ethyl alcohol. Platelets. M.p. 197–198°C. Yield 80 per cent. Analysis: Calculated for $C_{13}H_{17}O_4N_3$: N 15.05, found 14.86.

p-Aminobenzoyl-d,l-Leucine Amide.—Recrystallized from water. Platelets. M.p. 192–193°C. Yield 70 per cent. Analysis: Calculated for $C_{13}H_{19}O_2N_3$: N 16.87, found 17.05.

p-Nitrobenzoyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Platelets. M.p. 194–195°C. Yield 90 per cent. Analysis: Calculated for $C_{12}H_{13}O_6N_3$: N 14.24, found 14.36.

p-Nitrobenzoyl-Glycyl-Glycine Amide.—A solution of *p*-nitrobenzoyl-glycyl-glycine methyl ester in 150 parts of dry methyl alcohol was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. From here on the general procedure was followed. Recrystallized from 120 parts of 80 per cent ethyl alcohol. Long needles. Upon rapid heating m.p. 257–258°C. with decomposition. Yield 80 per cent. Analysis: Calculated for $C_{11}H_{12}O_5N_4$: N 20.0, found 19.98.

p-Nitrobenzoyl-Glycyl-Glycine Hydrazide.—10 gm. of finely ground *p*-nitrobenzoyl-glycyl-glycine methyl ester were suspended in 100 cc. of absolute ethyl alcohol and 20 cc. of 100 per cent hydrazine hydrate were added. After shaking for 1 hour the mixture became very thick and the crystal form of the substance

had changed from the long platelets of the ester to hair-like needles of the hydrazide. The hydrazide was filtered off, washed with absolute alcohol, and recrystallized from 140 parts of 30 per cent ethyl alcohol. Needles. M.p. 250–251°C. Yield almost quantitative.

p-Aminobenzoyl-Glycyl-Glycine Amide.—Recrystallized from 80 parts of absolute methyl alcohol. Platelets. M.p. 211–212°C. Yield 70 per cent. Analysis: Calculated for $C_{11}H_{14}O_3N_4$: N 22.40, found 22.52.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Methyl Ester.—Recrystallized from 50 per cent ethyl alcohol. Needles. M.p. 155–156°C. Yield 80 per cent. Analysis: Calculated for $C_{16}H_{21}O_6N_3$: N 11.97, found 12.20.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 10 parts of absolute ethyl alcohol and also from ethyl acetate containing 10 per cent alcohol. Irregular platelets. M.p. 178–179°C. Analysis: Calculated for $C_{15}H_{20}O_5N_4$ N 16.67, found 16.70.

p-Aminobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of water. Needles. Yield 80 per cent. Analysis: Calculated for $C_{15}H_{22}O_3N_4$: N 18.30, found 18.18.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent methyl alcohol. Needles. M.p. 177–178°C. Yield 70 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.67.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of 60 per cent ethyl alcohol. Platelets. M.p. 198–200°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.95.

p-Aminobenzoyl-Diglycyl-d,l-Leucine Amide.—The substance was dissolved in a small amount of absolute methyl alcohol and precipitated from this solution by addition of 10 volumes of dry ether. Amorphous. Shrinks at 137°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.38.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 60 parts of 30 per cent ethyl alcohol. Platelets. M.p. 154–155°C. Yield 90 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.55.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Needles. M.p. 201°C. Yield 75 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.62.

p-Aminobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 15 parts of absolute methyl alcohol. Platelets. M.p. 170–171°C. Yield 70 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.18.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 30 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 243–244°C. Yield 90 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 16.05.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Amide.—p-Nitrobenzoyl-tetraglycyl-d, l-leucine methyl ester was dissolved in 300 parts of boiling dry methyl alcohol and the solution was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. Then the general procedure was followed. Re-

crystallized from 30 parts of 50 per cent ethyl alcohol. Small needles. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.24.

p-Aminobenzoyl-Tetraglycyl-d,l-Leucine Amide.—Recrystallized from 30 parts of absolute methyl alcohol. No distinct crystalline form. Shrinks at 180°C. and melts at about 185°C. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{31}O_6N_7$: N 20.54, found 20.40.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from water. Rosettes of needles. Yield 75 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 15.98.

p-Nitrobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 216–217°C. Yield 80 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.18.

p-Aminobenzoyl-Diglycyl-d,l-Leucyl-Glycyl-Glycine Amide.—For purification the substance was dissolved in 15 parts of hot amyl alcohol. Amorphous substance which separated on cooling was filtered off and washed with dry ether. Yield 70 per cent. Analysis: Calculated for $C_{21}H_{31}O_6N_7$: N 20.54, found 20.38.

Azodyes.—The dyes used for inhibition tests were prepared by coupling the diazonium compounds, for ½ hour at 0–5°C., with an equimolar quantity of *m*-hydroxybenzoic acid in a solution kept alkaline by addition of a slight excess of sodium carbonate. After acidification and centrifugation the dyes were redissolved by means of dilute NaOH and after determination of the contents of dye stock solutions were made up to a concentration of 1:4 millimol of dye in 10 cc.

Immunization.—Rabbits were injected intravenously with 5 mg. of a suspension of horse azostromata (4) in 2 cc. After two to four courses of 6 daily injections each, with rest intervals of 1 week, the animals were bled on the 7th day following the last injection.

Tests.—Antigens used for the tests were made with chicken serum as described (2) using half as much of the diazonium compounds. The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The tests were observed for 1 hour at room temperature and then kept overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), ±, ∓, +, +±, ++, etc.

In the inhibition tests, the appropriate amount of the solutions had to be determined by preliminary experiments.

Precipitin and Absorption Tests

The precipitin reactions of immune sera for the four pentapeptides are presented in Tables I and II; they are in keeping with those exhibited by sera to shorter peptide chains in that cross reactions occurred chiefly, but not exclusively (*e.g.* G₄L serum No. 2 on G₅ or on LG₄) with peptides having the same amino acid at the carboxyl

TABLE I

To 0.2 cc. of 1:500 diluted stock antigens (prepared with chicken serum) were added 2 capillary drops of immune serum. The readings given were made after the tubes had remained for 1 hour at room temperature.

Immune sera	(Test antigens prepared from chicken serum and the aminobenzoyl derivatives of)

Immune sera	G	L	G₂	GL	LG	LL	G₃	G₂L	LG₂	GLG	G'	G₅	G₄L	G₂L	G₂LG₂	L₂G₂	LG'	Glut. G₅	Tyr. G₅	Glutathione
G₅	0	0	±	0	0	0	±	0	tr.	0	±	±	0	0	+	0	++	tr.	f. tr.	0
G₄L (No. 1)	0	±	0	++	0	0	0	++	0	0	0	0	+++	++	0	0	0	0	0	0
G₄L (No. 2)	±	f. tr.	+	+++	0	±	+	+++	0	0	+	+	+++	+++	f. tr.	0	+	0	±	tr.
G₂LG₂ (No. 1)	0	0	0	0	0	0	++	0	+	0	0	++	0	+++	+++	+	++	+	±	0
G₂LG₂ (No. 2)	tr.	0	0	0	tr.	0	++	tr.	±	0	0	±	0	++	++	tr.	++	f. tr.	+	0
L₃G₂	0	0	+	0	0	0	++	0	±	0	0	+	0	0	++	++	+	+	f. tr.	0

TABLE II

To 0.2 cc. of the stock antigens, in a dilution of 1:500 unless specified, were added 2 drops of immune serum. 1 hour readings are tabulated.

Immune sera	G	L	G₂	GL	G₂L	LG₂	G₂L 1:100	G₂L 1:500	G₂L 1:2500	G₂LG₂ 1:100	G₂LG₂ 1:500	G₂LG₂ 1:2500	G Am.	L Am.	G₂ Am.	GL Am.	G₂L Am.	LG₂ Am.	G₂L Am. 1:100	G₂L Am. 1:500	G₂L Am. 1:2500	G₂LG₂ Am. 1:100	G₂LG₂ Am. 1:500	G₂LG₂ Am. 1:2500
G₄L (No. 3)	0	tr.	0	±	++	0	±±	+++	+	±	0	+	0	0	0	0	0	0	±	0	0	tr.	0	±
G₂LG₂ (No. 2)	0	0	±	0	tr.	±	0	tr.	tr.	++	++	+	0	±	0	0	±	0	+	++	0	0	±	0
G₂L amide	0	0	0	0	0	0	0	tr.	0	0	0	0	0	0	0	+	±±	0	+	±±	±	+	0	±
G₂LG₂ amide	0	0	0	0	±	0	tr.	f. tr.	0	tr.	±±	±	0	0	0	±	+	0	±	+	f. tr.	+	±	±

end. The cross reactions were definitely related to similarities in constitution. For instance, the L_3G_2 immune serum gave precipitation with G_2, not with LG or GLG and the reactions of G_4L immune serum No. 2 increased in strength in the sequence L, GL, G_2L, etc. Or, G_5 immune serum precipitated G_2 but not LG and, G_3 much more than LG_2.

The antisera for G_4L Am. distinguished sharply between G_4L Am. and G_2LG_2 Am. (Table II) although they differ only in the

<div align="center">TABLE III</div>

To 0.04 cc. of the 1:100 diluted antigens were added 0.16 cc. absorbed immune serum (I.S.).

Two absorptions were carried out with G_5 azostromata, using 1.4 mg. with 1 cc. serum. With LG_2 the first absorption was made similarly, the second with half as much azostromata. The readings made after 15 minutes are shown in the first line, and those after 1 hour in the second line.

	G_2	LG_2	G_5	G_2LG_2
G_2LG_2 I.S. No. 1 absorbed with G_5	tr. tr.	± +	0 0	++ ++±
G_2LG_2 I.S. No. 1 absorbed with LG_2	± +	0 0	+ +±	++ +++

position of the leucine residue in the chain. Furthermore, the peptides and their corresponding amides in spite of great similarity in structure proved to be serologically different; G_4L and G_4L Am. showed weak, G_2LG_2 and G_2LG_2 Am. marked overlapping reactions. In addition cross precipitations were seen among amides as noted for peptides, however there was little overlapping between the two sorts of compounds. Of particular interest are observations that G_2LG_2 Am. sera react only faintly at best with LG_2 Am. antigen, which is identical as to the terminal part of G_2LG_2 Am., but do react (as to a lesser extent do G_2LG_2 sera) with G_2L Am., G_4L Am., and GL Am., which correspond to interior portions of the homologous substance. This is in contrast to the marked determinant influence of acid groups regularly encountered with free peptides, and the conclusion may be drawn that amide groups, though also strongly polar

rise to reactions with sera for the free peptides. However, these possibilities are ruled out by the following experiments. A serum for G_2LG_2 Am. was adsorbed with G_2LG_2 azostromata, and for control purposes with a non-reacting antigen as well, with the result that the antibodies which reacted with the amide were almost com-

TABLE VI

To 0.2 cc. of the given dilutions of stock antigen was added 1 capillary drop (in the case of G_4L amide antigen 3 drops) of immune serum either unabsorbed and diluted with normal rabbit serum or after being absorbed with G_4L Am. (2 mg. stromata per 1 cc. serum; 2 hours at room temperature). The readings given were made after 1 hour and after the tests had stood overnight in the ice box.

Immune serum G_4L (No. 2)	G_4L antigen						G_4L Am. antigen					
	1:50	1:100	1:200	1:400	1:800	1:1600	1:50	1:100	1:200	1:400	1:800	1:1600
After absorption with G_4L amide	0	±	++±	++±	++±	++	0	0	0	0	0	0
	0	±	++	++±	++±	++	f. tr.	f. tr.	f. tr.	0	0	0
Unabsorbed; diluted 3:4 in normal rabbit serum	tr.	±	++±	++	++	++±		+				
	tr.	+	++±	++±	++±	++		+±				
Unabsorbed; diluted 1:2 in normal rabbit serum	f. tr.	±	∓	+±	+±	+		∓				
	tr.	±	+	++	++±	+±		+				

pletely exhausted by the peptide antigen (Table V). (With another serum only a small amount of antibodies was left behind.) This proves that the bulk and not only a minor portion of the antibodies contained in the amide serum reacts with the free peptide. Then in the case of G_4L immune serum (Table VI) the reactions with G_4L Am. antigen disappeared almost completely upon absorption with G_4L Am. stromata, while the homologous reaction was hardly diminished and this, taken in conjunction with the persistence of the reaction for G_4L Am. upon dilution of the unabsorbed G_4L immune serum, shows that in the G_4L Am. antigen there is no appreciable amount of G_4L and that the sera contain a small fraction of a special antibody cross reacting with the amide.

TABLE VII a

For the inhibition tests 0.05 cc. of neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the stock chicken antigens, and homologous immune sera were then added (2 drops in the case of G_5 and 3 drops of G_4L and G_2LG_2); the concentrations of the inhibiting solutions (millimols in 10 cc.) were 1:8 for serum G_4L, 1:2 for serum G_2LG_2, and 1:4 for serum G_5. The control tube contained only antigen and immune serum.

Readings taken after 1 hour and after standing overnight.

	L	GL	G_2L	G_3L	G_5	G_4L	G_2LG_2	Control
G_4L I.S. (No. 4)	+	±	tr.	tr.	++	0	++	++
	++	+±	±	±	++++±	tr.	+++	+++±

	G_2	G_3	LG_2	G_4	G_5	LG_4	G_2LG_2	G_4L	Glut. G_2	Tyr. G_2	Control
G_5 I.S.	+	0	+	0	0	0	+	+	+	+±	++
	+±	±	+±	tr.	0	tr.	+±	+±	++	++	+++
G_2LG_2 I.S. (No. 3)	+	+	tr.	+	+	+	0	+±	+	+±	++
	+++±	+++±	+++	+++±	+++±	+++±	0	+++±	+++±	+++	+++±

TABLE VII b

For the inhibition tests 0.05 cc. of solutions of peptide amide azodyes (concentration 1:33 millimol in 10 cc.) were mixed with 0.2 cc. of 1:500 dilutions of the stock G_4L Am. chicken antigen, and 2 capillary drops of immune serum were then added. Readings are given after 15 minutes and 1 hour, and after standing overnight.

	G Am.	L Am.	G_3 Am.	GL Am.	G_2L Am.	LG_2 Am.	G_4L Am.	G_2LG_2 Am.	G_4L	Control
G_4L amide I.S.	+±	+	+±	±	±	+±	0	+±	+	+±
	++	+±	++	±	±	++	tr.	++	+±	++
	+++±	+++±	+++±	+±	+±	+++±	tr.	+++±	+++±	+++±

Inhibition Tests

Inhibition tests were carried out with nitrobenzoylated peptides;[*] peptide amides because of their poor solubility were converted into

[*] Positive inhibition reactions were also obtained with higher concentrations of non-acylated G_2LG_2. The compounds G_5, L_3G_2, G_4L, NO_2-benzoyl L_4G_2 could not be tested because of insufficient solubility.

easily soluble azodyes by diazotizing the aminobenzoyl derivatives and coupling to *m*-hydroxybenzoic acid. Results are presented in Tables VII *a* and VII *b*. When it is taken into account that only those substances are included which in the form of azoproteins gave positive precipitin reactions with the sera in question, these tests are

TABLE VIII

For the inhibition tests neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the heterologous antigens before addition of the immune sera. In the case of G_4L serum, 0.05 cc. of solutions containing 1:8 millimol of the peptides in 10 cc. were tested, with G_2LG_2 serum, 0.1 cc. of solutions containing 1:12 millimol in 10 cc. 2 drops were used of G_4L and 3 drops of G_2LG_2 immune sera.

Readings taken after 1 hour and after standing overnight.

	L	GL	G_2L	G_5	G_4L	Control
G_4L I.S. No. 3 on G_2L antigen	+ ++	± ±	tr. ±	+± ++±	0 tr.	+± ++±

	G	G_2	G_2L	LG_2	G_5	G_2LG_2	LG_4	Control
G_2LG_2 I.S. No. 4 on G_2 antigen	+ +±	± +	+ +±	± +	± +	0 tr.	± +	+ +±

rather strikingly specific for the homologous haptens. The distinction between the sera G_5 and G_2LG_2 differing in only one amino acid, may be mentioned and, again, the shading off of the reactions of sera G_4L, in the order G_4L, G_3L, G_2L, GL, and L (Table VII *a*). The amide azodyes did not inhibit the reactions of the peptide immune sera but showed sharply specific inhibitions with the homologous amide sera (Table VII *b*). A definite reaction was also seen with G_2LG_2 Am. serum and G_2LG_2 used either as nitrobenzoyl derivative or as azodye.

The inhibition method proved to be of advantage for characterizing the nature of antibodies operative in the cross precipitin reactions with heterologous antigens.

From such experiments in part presented for illustration (Table VIII), it appears that also those antibodies that produce precipitation with heterologous antigens are specifically related to the homologous substance in its entirety (page 713), that is to the one which served as determinant in the formation of the antibodies. When, therefore, one antigen is precipitated by several different immune sera, it can be shown by inhibition tests that the antibodies concerned are different, as in the instances given in Table IX.

TABLE IX

The procedure for the inhibition tests was that described in Table VII a; the concentrations of the inhibiting solutions were (as millimols in 10 cc.) 1:2, 1:8, and 1:16 as employed respectively with the three immune sera listed. 2 drops each were used of G_5 and G_2LG_2, and 3 drops of G_3 immune sera.

The readings presented were made after 15 minutes, 1 hour, and after standing overnight.

	G_2	G_5	G_2LG_2	Control
G_3 I.S. on G_3 antigen	0	<u>tr.</u>	+	++
	f. tr.	<u>tr.</u>	+	++±
	f. tr.	<u>tr.</u>	+±	++±
G_5 I.S. on G_3 antigen	tr.	0	+	+±
	+	tr.	+±	++
	+	±	++	++±
G_2LG_2 I.S. No. 2 on G_3 antigen	tr.	tr.	0	+
	+	±	0	+±
	+	±	0	+±

COMMENT

The four pentapeptides examined, although all contained only glycyl and leucyl residues, were distinct in their precipitin reactions when tested with various antigens; and replacement of even one of five glycines by leucine resulted in a noticeable alteration in serological properties. A pronounced serological change was brought about by the conversion of peptides into amides, and a similar modification was observed upon esterification of peptides (6).

The specificity of the sera was more conspicuous in inhibition than

in precipitin tests. The two kinds of reactions cannot readily be compared quantitatively, yet the apparently greater specificity of inhibition tests may be explained by the consideration that in inhibition reactions there is a competition between the weak affinity of the immune sera for heterologous haptens and the strong affinity to the homologous antigen, while in precipitin reactions there is present only one reacting substance and, moreover, precipitins of low activity can be aided and carried down in the precipitate by more potent antibodies (Heidelberger).[3]

From the absorption experiments described above one can conclude that the specificity of antibodies engendered by pentapeptides may be directed towards the molecule as a whole. In agreement with this, greater or at least equal inhibition of cross reactions was produced by the haptens homologous to the immune sera in comparison with those corresponding to the heterologous antigens tested (4). No evidence was found to demonstrate the formation of separate antibodies to several parts of the peptide structure, as had been observed with some other compounds in which highly determinant acid groups were linked to, and separated by a benzene ring (8). The difference in constitution between compounds of this sort and —CO—NH— chains may possibly be the reason for the disparity in the character of the antibodies formed. While thus it appears that antibodies may be formed which are specific for peptide chains consisting of five amino acid residues, it will be one of the next tasks to investigate, with the use of higher polypeptides, how large a structure in its entirety may be reflected in the configuration of antibodies, a question of considerable significance for the serological specificity of proteins.

The overlapping reactions of the peptide sera here reported were in general in accord with those formerly observed but a new fact emerged from the reactions of immune sera to a peptide amide (diglycyl-leucyl-diglycyl amide), namely strong cross reactions involving not the end groups but other parts of the molecule, a result likewise to be

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[3] The lesser specificity reported for inhibition reactions with very simple compounds, *e.g.* substituted benzoic acids (7), may be ascribable to the circumstance that the "homologous" haptens did not so closely correspond to the antigens used in which the azodye structure probably plays a greater part in determining the reactivity than in the more complicated instances.

considered in protein reactions. These sera, unlike those for the corresponding free peptide, gave no or only weak overlapping reactions with peptide amides identical in their terminal groups with the homologous substance. It would seem, therefore, that the serological predominence of carboxyl groups (see also Goebel, 9) is suppressed by conversion into amides and that there is a definite difference in the serological significance of strongly polar amides and of dissociating acid groups. This may suggest that the combinations occurring in antigen-antibody reactions are not all of the same kind.

SUMMARY

Experiments are described dealing with immune sera to pentapeptides and peptide amides. Absorption and inhibition tests gave no indication of the presence in the immune sera of special antibodies for portions of a peptide molecule but the antibodies appeared to be specific for an entire pentapeptide even though the sera contained qualitatively different fractions. Marked disparity was found between the reactions of peptides and corresponding amides indicating differences between acid and other polar groups in their influence on serological specificity.

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BLOOD PLASMA PROTEIN PRODUCTION AS INFLUENCED BY AMINO ACIDS

CYSTINE EMERGES AS A KEY AMINO ACID UNDER FIXED CONDITIONS

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When one studies the production of plasma protein or hemoglobin or any other body protein inevitably there follows a consideration of the building stones of such protein. One always cherishes the hope (perhaps the illusion) that some key amino acid or group of amino acids may be found. A good many amino acids can influence the building of hemoglobin under uniform conditions in experimental anemia (13) but no one acid or small group of amino acids as yet appears to stand out above all others in this reaction. It appears that *cystine* in the experiments below nearly qualifies as a *key amino acid* in the complicated metabolic reaction which must be responsible for the construction of plasma protein in the depleted dog. Cystine alone as a supplement to gelatin does not render this incomplete protein a potent factor in plasma protein building. Cystine plus tyrosine or tryptophane as a supplement to gelatin makes this mixture as potent as beef serum itself for the building of new plasma protein in the hypoproteinemic dog.

This paper continues the study of blood plasma protein production in dogs as influenced by various amino acids alone or as supplements to the incomplete protein gelatin. *Plasma protein production* is measured by the determination of the protein removed by daily *plasmaphereses* (removal of whole blood by vein puncture and return of washed red cells suspended in a saline solution). Many factors which influence plasma protein regeneration have been discussed and the related data published in previous reports (3, 9, 7, 6, 5).

Methods

The procedures used in obtaining the data reported here are those fully described or referred to in a previous communication (5). It may be repeated here that the gelatin used in the experiments is a white, granular gelatin (Will Corporation) containing by Kjeldahl analysis 15.1 per cent nitrogen (83.8 per cent protein (4)). The pure crystalline amino acids used (Eastman Kodak Company) are the naturally occurring types except for racemic mixtures of phenylalanine and of methionine.

EXPERIMENTAL OBSERVATIONS

The data reported in this paper have been obtained from experiments with two dogs, one of which was observed for over a year and the other for 5 months. Tables 1 and 1-*a*, 2 and 2-*a* give these data for 54 consecutive weekly periods as recorded in dog 36-196. Tables 3 and 3-*a* record 23 similar periods for dog 37-6. Clinical details and the exact make-up of the diets given during each period are recorded in the Clinical Experimental Histories which follow. It is to be noted that raw *pork liver* was the *only protein given* these dogs except for the occasional test supplements of gelatin.

In the process of reducing the concentration of circulating plasma protein in dog 36-196 from a normal level of 5.70 per cent to the desired low level of 3.80 to 4.00 per cent, about 49 gm. plasma protein were removed during the first 3 periods of plasmapheresis (Table 1). It is estimated that the liver basal diet fed during 2 of these 3 periods furnished the materials to produce 24 of the 49 gm. The remaining 25 gm. must have come from materials (*a reserve store*) already present in the body and readily available for conversion into plasma protein. If we estimate the entire *reserve store* of plasma protein building material as *determined by the first 7 periods*, we note a total output of 116 gm. and a basal output for the 6 periods in which the basal diet was fed of 72 gm. (disregarding the gelatin feeding). This gives about 40 gm. as the reserve store. The size of this reserve store in any given dog depends largely upon the quantity and quality of dietary protein in the few weeks preceding plasmapheresis.

The *basal output of plasma protein* is determined by averaging those liver basal periods which are neither high nor low on account of some immediately preceding test. Thus, in dog 36-196 (Tables 1 and 2), the average of periods 15 to 19, 22, 26, 28, 30, 33, 35, 43, 46, 48, and 50 is 12 gm.

Of the 18 odd grams of protein removed above this 12 gm. basal output during periods 4 to 7, how much is reserve store and how much if any is due to the supplements of *tryptophane* and of *gelatin plus tryptophane* is not certain. In an earlier report (5) tryptophane alone produced no appreciable increase in plasma protein production but gelatin plus tryptophane was potent in a ratio of 3 gm. of gelatin protein to produce 1 gm. of plasma protein. No such potency is evident in this test, nor when repeated (period 10) nor when tried in another dog (Table 3, period 16).

The low production evident during periods 9 to 12 is not well explained. The animal appeared normal but may have been affected by the very hot summer weather. Period 13 gives confirmation of the observation previously reported (5) that the dog with exhausted reserve stores of plasma protein building material can make little if any new plasma protein while consuming a *protein-free* otherwise adequate diet. The one gram listed as "protein" intake (period 13) is probably only in part true protein suitable for utilization in nutrition. The expected fall in urinary nitrogen is noted (Table 1-*a*). It is important to note that when the liver basal diet was again fed in the following week (period 14) the urinary nitrogen did not return to the basal level nor did the plasma protein output rise to the basal level. It may be proper to argue that the fasting week depleted body protein whose repair or replacement took precedence over the manufacture of new plasma protein.

Gelatin is lacking in *tryptophane* and *tyrosine* and is very poor in *cystine* (1). When it is supplemented with these three amino acids, as in period 20, the whole mixture becomes a potent source of materials for plasma protein production—slightly less than 3 gm. gelatin protein being required to produce 1 gm. plasma protein. This combination is practically as potent in plasma protein production as any substance yet examined—2.6 gm. beef serum yielded 1 gm. plasma protein (9). It should be noted that often there is a *carry over* of the effect of supplementary feeding upon plasma production into the week (period 21) or two following such feeding.

It is instructive to contrast periods 6 and 10 in which there is gelatin plus tryptophane added to the basal diet, with periods 20 and 23 (Table 1) in which gelatin is supplemented with cystine and tyro-

TABLE 1

Blood Plasma Protein Regeneration

Gelatin Becomes Very Efficient upon Addition of Cystine, Tyrosine, and Tryptophane

Dog 36-196.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Protein removed above basal*	Blood plasma Average concentration		R.B.C. hematocrit, average	Plasma volume
					Total protein	A/G ratio		
		gm.	gm.	gm.	per cent		per cent	cc.
	Kennel				5.70	1.59	41.7	—
1	Fasting	0	4.8		5.68	1.33	41.1	490
2	Liver basal	70	26.4		4.90	1.12	43.9	460
3	Liver basal	70	18.0		4.13	0.76	47.6	464
4	Liver basal + tryptophane, 2.0 gm.	70	16.6		4.10	0.94	50.3	450
5	Liver basal	70	15.7		4.11	1.01	50.0	439
6	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	129	17.8	2.0±	4.15	1.02	48.1	474
7	Liver basal	70	16.5		4.05	0.97	50.3	441
8	Liver basal	70	11.9		3.95	0.85	51.7	467
9	Liver basal	70	7.3		4.03	0.97	53.6	385
10	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	129	11.8	0.0	4.05	0.93	54.3	456
11	Liver basal	70	9.9		3.84	0.83	49.7	478
12	Liver basal	70	9.1		4.02	0.80	46.2	496
13	Protein-free	1±	1.7		3.95	—	45.4	444
14	Liver basal	70	4.7		3.95	0.70	41.7	474
15	Liver basal	70	12.2		4.06	0.74	46.5	437
16	Liver basal	70	10.5		3.87	0.79	50.4	447
17	Liver basal	70	10.3		4.20	0.74	45.5	—
18	Liver basal	70	14.5		3.99	0.84	45.7	424
19	Liver basal	70	10.0		3.81	0.73	47.2	452
20	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm. + cystine, 4.2 gm. + tyrosine, 4.2 gm.	129	25.0	20.1	4.15	0.91	47.6	465
21	Liver basal	70	20.5		3.86	0.81	49.2	460
22	Liver basal	70	10.6		3.80	0.75	47.0	418
23	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + cystine, 4.2 gm.	129	18.1	23.0	4.08	0.91	45.6	471
24	Liver basal	70	22.4		4.15	0.67	49.4	418
25	Liver basal	70	18.6		3.88	0.80	52.6	492
26	Liver basal	70	11.9		3.79	0.73	50.9	492
27	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm.	129	15.1	3.0	3.99	0.79	49.3	419
28	Liver basal	70	11.9		3.97	0.74	48.9	419

* Estimated basal output per week equals 12 gm. plasma protein.

TABLE 1-a

Weight and Nitrogen Balance

Dog 36-196.

Period 7 days	Diet	Weight	Nitrogen balance					Intake minus output
			Intake		Output			
			in diet	in excess R.B.C. injected	in plasma	in urine	in feces	
		kg.	gm.	gm.	gm.	gm.	gm.	gm.
	Kennel	10.5						
1	Fasting	9.1	0.0	0.0	0.8	14.8	0.0	−15.6
2	Liver basal	9.2	11.2	8.0	4.2	12.6	3.1	−0.7
3	Liver basal	9.2	11.2	4.3	2.9	11.3	3.1	−1.8
4	Liver basal + tryptophane, 2.0 gm.	9.2	11.5	3.5	2.7	11.1	2.0	−0.8
5	Liver basal	9.0	11.2	−2.3	2.5	9.6	1.8	−5.0
6	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	9.0	22.2	5.2	2.8	19.5	2.3	+2.8
7	Liver basal	9.2	11.2	2.9	2.6	10.3	1.7	−0.5
8	Liver basal	9.3	11.2	1.6	1.9	12.3	2.3	−3.7
9	Liver basal	9.3	11.2	−1.6	1.2	9.6	2.0	−3.2
10	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	9.5	22.2	4.4	1.9	18.3	2.3	+4.1
11	Liver basal	9.5	11.2	1.4	1.6	10.6	2.3	−1.9
12	Liver basal	9.6	11.2	−0.6	1.5	9.5	3.3	−3.7
13	Protein-free	9.4	0.3	0.0	0.3	6.5	1.6	−8.1
14	Liver basal	9.5	11.2	1.6	0.8	7.8	1.0	+2.8
15	Liver basal	9.6	11.2	4.1	2.0	8.0	2.5	+2.8
16	Liver basal	9.7	11.2	−0.7	1.7	8.8	2.0	−2.0
17	Liver basal	9.6	11.2	2.2	1.7	9.8	3.1	−1.2
18	Liver basal	9.6	11.2	2.6	2.3	9.0	2.1	+0.4
19	Liver basal	9.5	11.2	4.5	1.6	9.3	2.7	+2.1
20	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm. + cystine, 4.2 gm. + tyrosine, 4.2 gm.	9.7	23.0	3.4	4.0	12.5	2.8	+7.1
21	Liver basal	9.5	11.2	3.7	3.3	9.2	2.7	−0.3
22	Liver basal	9.5	11.2	1.5	1.7	9.8	2.6	−1.4
23	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + cystine, 4.2 gm.	9.3	22.6	1.5	2.9	14.4	3.3	+3.5
24	Liver basal	9.3	11.2	3.8	3.6	8.1	2.2	+1.1
25	Liver basal	9.3	11.2	1.5	3.0	9.0	1.8	−1.1
26	Liver basal	9.3	11.2	1.7	2.0	8.6	—	—
27	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm.	9.3	22.1	4.7	2.5	15.5	2.4	+6.4
28	Liver basal	9.2	11.2	2.4	2.0	9.1	2.1	+0.5

sine.   In the first two experiments (periods 6 and 10) there is no excess production of new plasma protein and there is a large excess of urinary nitrogen (19.5 and 18.3 gm.).   In the second two experiments (periods 20 and 23) there is a large surplus of new plasma protein (20 to 23 gm.) but a decrease in urinary nitrogen (12.5 and 14.4 gm.).   It seems inevitable to conclude that the gelatin incompletely supplemented presents a mélange of amino acids which cannot be utilized to build plasma protein or other body protein and are thrown away in the urine.   When cystine and tyrosine or tryptophane are added to the gelatin, the body is then able to utilize the gelatin split products in an extraordinarily efficient manner so that less nitrogen appears in the urine and much more plasma protein is manufactured.

During periods 23 to 28 (Table 1) and 29 to 35 (Table 2) 5 different groupings of these amino acids with gelatin were tried.   It appears that the addition to gelatin of *cystine* and either or both *tryptophane* and *tyrosine* gives a mixture highly efficient for plasma protein production.   Without added cystine the gelatin is inefficient; yet cystine alone does not appreciably improve the efficiency.   Corroboration of some of these findings is given in another dog (Table 3, periods 14 to 21) and the results of all experiments are summarized in Table 4.

Tomatoes were added to the basal diet in period 36 (Table 2) in an attempt to duplicate the conditions under which tryptophane and gelatin produced a large amount of new plasma protein (29 gm.) in the earlier experiment referred to above (5).   No significant production of protein above the basal output was obtained.   The shock reaction occurring on the 4th day of the 37th period prevented accurate measurement of any hypothetical carry over but no evidence of any such impending reaction was given in those first 4 days.

The *anaphylactic shock* reaction is part of another problem not considered in this report.   The dog recovered from the severe effects in two days but in order to insure a full return to a normal state the protein intake in the diet was doubled and plasmapheresis was discontinued (periods 38 to 40, see Clinical History).   Plasmapheresis was resumed in the 41st period and it required 2 weeks to reestablish basal conditions as obtained in the 43rd period.   It is obvious that protein had been stored during periods 38 to 40—a weight gain of 0.9 kg. and a large positive nitrogen balance speak for this.   Also,

during the 6 periods 38 to 43 only 78.8 gm. protein were removed to achieve basal conditions, whereas had there been no call for protein to repair shock injury it would probably have been necessary to remove 108 gm. (5).

Cystine and tyrosine were again added with gelatin to the liver basal in period 44. While the response in plasma protein production was not so large as in period 23, it compares favorably and indicates consistent performance of this biological test machine.

Laked red blood cells afford little if any material for plasma protein formation when introduced into the blood stream. This conclusion had already been reached from experiments in normal dogs (10) and is confirmed in this plasma depleted dog in period 47. Slightly less than 25 gm. of hemoglobin containing about 23 gm. protein was responsible for the production of 1 gm. of plasma protein, a quantity well within the limits of error in this type of experiment. These experiments do not support recent inferences by Melnick, Cowgill, and Burack (8) to the effect that hemoglobin may be utilized to build new plasma protein.

Methionine adds no more to the efficiency of gelatin than cystine alone and cannot replace cystine in its potent combination with tyrosine (periods 49 to 52). Of considerable interest is the apparent inability of phenylalanine to act effectively with cystine and gelatin in the way that tyrosine does (period 53).

In Tables 1-*a* and 2-*a* it should be noted that throughout the entire year the animal's weight varied little—between 8.8 and 9.7 kg. except for the loss during the anaphylactic shock episode. Moreover, for the entire 54 weeks a positive nitrogen balance of $14 \pm$ gm. was obtained. The urinary nitrogen figures are of some special interest. For the 15 basal periods listed above in determination of the *basal protein output* the average weekly urinary nitrogen excretion was 8.8 gm., with limits of 7.4 and 9.8 gm. If we compare these figures (8.8 gm. nitrogen per week) during the *basal diet periods* with the figures observed during *gelatin feeding* there are striking differences. When gelatin plus tryptophane or tyrosine or both were added to the basal ration the urinary nitrogen per week averaged 17.6 gm. and the plasma protein output 1 to 2 gm. In striking contrast when gelatin plus cystine and tyrosine or tryptophane were added to the

TABLE 2 (*continues Table 1*)

Methionine and Phenylalanine Ineffective Compared with Cystine and Tyrosine
Hemoglobin by Vein Not Utilized to Build Plasma Protein

Dog 36-196.

Period 7 days	Diet	Protein intake Total for 7 days	Plasma protein removed Total for 7 days	Protein removed above basal*	Blood plasma Average concentration		R.B.C. hematocrit, average	Plasma volume
					Total protein	A/G ratio		
		gm.	gm.	gm.	per cent		per cent	cc.
29	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm.	129	15.9	5.7	4.07	0.80	49.5	442
30	Liver basal	70	13.8		3.98	0.69	49.5	425
31†	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + tryptophane, 2.8 gm.	129	21.5	18.8	4.29	0.88	49.3	428
32	Liver basal	70	22.5		4.13	0.79	50.5	389
33	Liver basal	70	10.8		3.78	0.67	49.9	431
34	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + tryptophane, 2.8 gm.	129	12.4	0.7	3.88	0.62	48.8	434
35	Liver basal	70	12.3		3.87	0.58	46.6	447
36	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm. + tomatoes, 245 gm.	132	13.7	1.7+	3.91	0.55	47.4	376
37	Liver basal (anaphylactic shock)	58	8.0		3.95	0.55	46.5	434
38	Liver basal + liver, 350 gm.	140	2.8		4.65	0.57	43.2	470
39	Liver basal + liver, 350 gm.	140	17.5		5.05	0.67	44.5	452
40	Liver basal + liver, 350 gm.	140	5.0		4.97	1.41	43.8	469
41	Liver basal	70	26.1		4.32	0.77	43.6	471
42	Liver basal	70	14.8		3.69	0.56	46.0	484
43	Liver basal	70	12.6		3.85	0.55	46.9	450
44	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + cystine, 4.2 gm.	129	20.5	14.6	4.06	0.68	47.2	442
45	Liver basal	70	17.3		3.74	0.72	45.0	461
46	Liver basal	70	12.8		3.84	0.62	45.5	460
47	Liver basal + hemoglobin by vein, 24.8 gm.	93	13.1	1.0	3.89	0.49	44.9	419
48	Liver basal	70	11.9		3.74	0.60	44.1	486
49	Liver basal + gelatin, 70 gm. + methionine, 5.2 gm.	129+	14.4	4.0	3.92	0.69	46.1	477
50	Liver basal	70	13.6		3.90	—	47.4	437
51	Liver basal + gelatin, 70 gm. + methionine, 5.2 gm. + tyrosine, 4.2 gm.	129	15 6	6.8	3.97	0.70	46.2	483
52	Liver basal	70	15.2		3.86	0.65	45.7	471
53	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + phenylalanine, 4.2 gm.	129	15.4	3.5	3.93	0.64	46.2	473
54	Liver basal	69	12.1		3.85	0.72	47.7	429

* Estimated basal output per week equals 12 gm. plasma protein.

† Fed 60 gm. gelatin—recalculated on basis of 70 gm.

TABLE 2-*a* (*continues Table 1-a*)

Weight and Nitrogen Balance

Dog 36-196.

Period 7 days	Diet	Weight	Nitrogen balance Intake in diet	Nitrogen balance Intake in excess R.B.C. injected	Nitrogen balance Output in plasma	Nitrogen balance Output in urine	Nitrogen balance Output in feces	Intake minus output
		kg.	gm.	gm.	gm.	gm.	gm.	gm.
29	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm.	9.2	22.2	3.7	2.6	13.5	3.1	+6.7
30	Liver basal	9.2	11.2	1.1	2.3	8.4	2.5	−0.9
31*	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + tryptophane, 2.8 gm.	9.2	22.6	2.2	3.5	12.6	2.4	+6.3
32	Liver basal	9.1	11.2	0.6	3.7	10.6	1.7	−4.2
33	Liver basal	9.1	11.2	0.8	1.8	9.7	2.3	−1.8
34	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + tryptophane, 2.8 gm.	8.9	22.5	1.7	2.1	17.7	3.0	+1.4
35	Liver basal	8.9	11.2	2.2	2.0	9.3	2.4	−0.3
36	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm. + tomatoes, 245 gm.	8.8	22.7	2.4	2.3	17.0	2.3	+3.5
37	Liver basal (anaphylactic shock)	8.2	9.3	1.1	1.3	13.0	1.1	−5.1
38	Liver basal + liver, 350 gm.	8.6	22.4	0.7	0.5	10.2	3.3	+9.1
39	Liver basal + liver, 350 gm.	8.9	22.4	3.7	2.8	11.6	2.7	+9.0
40	Liver basal + liver, 350 gm.	9.1	22.4	0.5	0.8	11.3	2.7	+8.1
41	Liver basal	8.9	11.2	6.0	4.3	11.0	2.5	−0.6
42	Liver basal	9.1	11.2	1.9	2.5	9.5	2.8	−1.7
43	Liver basal	8.9	11.2	1.9	2.0	9.1	2.9	−0.9
44	Liver basal + gelatin, 70 gm. + tyrosine, 4.2 gm. + cystine, 4.2 gm.	8.8	22.6	−0.7	3.4	13.3	3.4	+1.6
45	Liver basal	8.8	11.2	1.3	2.9	8.2	2.8	−1.4
46	Liver basal	9.0	11.2	1.4	2.1	7.4	2.8	+0.3
47	Liver basal + hemoglobin by vein, 24.8 gm.	8.9	11.2	3.6†	2.2	9.8	2.9	−0.1
48	Liver basal	9.0	11.2	1.0	2.0	8.7	2.8	−1.3
49‡	Liver basal + gelatin, 70 gm. + methionine, 5.2 gm.	9.0	22.2+	2.0	2.4	13.4	3.6	+4.8
50	Liver basal	9.1	11.2	−2.0	2.3	7.7	3.5	−4.3
51	Liver basal + gelatin, 70 gm. + methionine, 5.2 gm. + tyrosine, 4.2 gm.	9.2	22.5	1.3	2.6	13.4	3.2	+4.6
52	Liver basal	9.0	11.2	0.1	2.5	8.5	2.9	−2.6
53	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + phenylalanine, 4.2 gm.	9.2	22.6	1.9	2.6	13.4	3.2	+5.3
54	Liver basal	9.2	11.2	0.3	2.0	9.8	4.5	−4.8
	Totals for 54 periods (Tables 1-a and 2-a)..		762.1	106.0	123.5	593.0	134.4	+14.3

* See footnote to period 31, Table 2. The figures for nitrogen balance are correspondingly adjusted.

† Includes nitrogen in laked red blood cells injected intravenously.

‡ The dog consumed additional protein—see Clinical Experimental History.

basal ration the urinary nitrogen per week averaged 13.2 gm. and the plasma protein output 19 gm. We may say that about 4.4 gm. of nitrogen was conserved from wastage in the urine which might account for about 26 gm. of new body protein and actually 19 gm. appeared as plasma protein. It should be noted also that when gelatin was supplemented with cystine or methionine the urinary nitrogen averaged about 13.4 gm. per week although the plasma protein output was only 4 to 7 gm. per week. This may suggest some amino acid conservation in this dog with the production of some body protein but only a modest amount of plasma protein.

Clinical Experimental History.—Dog 36-196 (Tables 1 and 1-*a*; 2 and 2-*a*). An adult female beagle hound, weighing 10.5 kg., was fasted for one week. It was then given a liver basal diet which it consumed daily for the next 54 weeks except as herein noted. The diet consisted of raw pork liver, 50 gm.; cane sugar, 50 gm.; cornstarch, 50 gm.; cod liver oil, 25 gm.; salt mixture, 2 gm.; bone ash, 10 gm.; kaolin, 5 gm. When this basal diet was supplemented by gelatin the basal caloric intake was maintained by reducing the cane sugar given to 41 gm. The "protein-free" diet fed during the 13th period contained cane sugar, 77 gm.; dextrin, 25 gm.; lard, 23 gm.; butter fat, 9 gm.; salt mixture, 2 gm.; bone ash, 3 gm.; "Ryzamin B" concentrate, 0.4 gm. (*i.e.*, 32 international units of vitamin B_1); liver extract, parenteral,[1] 1 cc.; White's cod liver oil concentrate, 3 tablets (*i.e.*, about 9400 units of vitamin A and 940 units of vitamin D). Consumption of these diets was uniformly complete, except during period 37. The dog was sensitized to horse serum and when the shocking dose was given in the 37th period a severe characteristic reaction occurred. 3 weeks (38 to 40) were allowed for recovery from this disturbance, during which time the intake of liver in the diet was doubled to 100 gm. daily. Segments of *Tenia pisiformis* appeared in the feces and arecoline hydrobromide, 18 mg., was given in period 38, yielding 7 scolices and their bodies. Plasmapheresis, resumed in the 38th week after a 10 days' rest, was not performed in the 40th week. The clinical condition of the dog was good.

The *hemoglobin* given intravenously during period 47 was prepared from fresh washed red blood cells (dog) laked in distilled water. The three injections given each day were at least 6 hours apart and the exact quantities of hemoglobin given, as determined by the acid hematin method, varied from 0.97 to 1.66 gm. After 5 of the 19 injections there was evidence of slight hemoglobinuria both grossly and by benzidine test; during and after the other 14 injections the benzidine test was negative. Clinically the dog gave not the slightest reaction.

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[1] We are indebted to Eli Lilly and Company for this material.

During period 49 the dog consumed about 5 gm. additional protein in ground beef and salmon bread inadvertently available. At most this quantity of these proteins could produce only 1 gm. plasma protein, and such an allowance has been made in calculating the percentage utilization of gelatin plus methionine given in Table 4.

In Tables 3 and 3-a observations are recorded on another dog (37-6) tested under similar conditions and with some of the same amino acid and gelatin combinations. as noted in the experiments tabulated above. The experiments in the two dogs are in complete agreement. This dog (37-6, Tables 3 and 3-a) had a rather large reserve store of plasma protein building material which required 11 weeks for complete removal. During the 11 periods including the first period of fasting, the dog put out 270.7 gm. of plasma protein and if we deduct 190 gm. for the 10 weeks of basal diet intake we record 80 gm. as the reserve, using 19 gm. as the basal weekly plasma protein output. During these periods (2 to 11 inclusive) the diet intake was 84 gm. of protein and the basal diet was determined on subsequent periods on a protein intake of 70 gm. (Table 3). If we correct for this larger protein intake the basal output for periods 2 to 11 should have been 22.8 gm. plasma protein. Using this figure the reserve store of plasma protein building material would be 43 gm. but the anaphylactic shock comes into the picture. With anaphylactic shock the body proteins suffer injury and there is an excess of nitrogen eliminated in the urine (not shown in Table 3-a but refer to Table 2-a). This may well signify removal of protein material from the reserve stores and to explain an increase in urinary nitrogen of 4 gm. would require 24 gm. of body protein. The true figure of plasma protein reserve store probably falls between 43 and 80 gm. for this dog.

The estimate of the *basal output* of plasma protein in dog 37-6 on a protein intake of 70 gm. per week is an average of periods 12, 13, 15, and 20 or 18.8 gm.

Protein utilization was very efficient in dog 37-6 (Tables 3 and 3-a), much more so than in the other dog (36-196). On the same diet and the same protein and caloric intake this dog (37-6) was able to gain slightly in weight, produce more new plasma protein, and eliminate less urinary nitrogen. Both dogs were in nitrogen equilibrium. This dog (37-6) required only 4 gm. of diet protein to produce 1 gm.

TABLE 3
Blood Plasma Protein Regeneration
As Modified by Gelatin with Tryptophane, Cystine, and Phenylalanine

Dog 37-6.

Period 7 days	Diet	Protein intake Total for 7 days (gm.)	Plasma protein removed Total for 7 days (gm.)	Protein removed above basal* (gm.)	Blood plasma Average concentration — Total protein (per cent)	A/G ratio	R.B.C. hematocrit, average (per cent)	Plasma volume (cc.)
1	Kennel				5.69	1.71	44.8	478
2	Fasting	0	29.2		5.24	—	48.7	—
3	Liver basal	84	28.4		4.16	0.95	49.3	414
4	Liver basal	84	23.1		3.95	1.02	46.8	438
5	Liver basal (anaphylactic shock)	84	22.1		4.03	0.98	47.6	423
6	Liver basal	72	19.5		4.22	1.19	46.1	557
7	Liver basal	84	29.1		4.30	0.78	45.0	434
8	Liver basal	84	27.4		4.08	0.84	47.4	419
9	Liver basal	84	24.0		3.83	0.81	47.5	433
10	Liver basal	84	24.5		3.95	0.91	45.6	468
11	Liver basal	84	19.2		3.99	0.82	46.8	472
12	Liver basal	70	24.2		4.02	0.84	47.3	442
13	Liver basal	70	19.9		3.88	0.85	48.7	434
14	Liver basal + gelatin, 69 gm.	127	16.9		3.90	0.71	48.3	410
15	Liver basal	70	16.9	1.0±	3.85	—	45.6	444
16	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	129	18.8		3.93	0.79	46.8	460
17	Liver basal	70	18.7	2.0	3.97	0.86	47.4	481
18	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + tryptophane, 2.8 gm.	129	21.3		4.03	0.76	44.6	477
19	Liver basal	70	25.7	24.0	4.32	1.16	46.1	—
20	Liver basal	70	31.0		4.51	0.72	47.2	431
21	Liver basal	70	23.5		3.99	0.89	46.0	462
22†	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + phenylalanine, 4.2 gm.	129	19.8		4.03	0.81	46.8	431
23	Liver basal	70	22.5	7.5	4.01	0.61	47.6	—
		70	23.0		4.11	—	46.6	--

* On a protein intake of 70 gm. the estimated basal output of plasma protein equals 19 gm. per week.

† One day's diet was omitted from this period. For comparative purposes, the figures given for protein intake and output are 7/6 of those obtained.

TABLE 3-*a*

Weight and Nitrogen Balance

Dog 37-6.

Period 7 days	Diet	Weight	Nitrogen balance					
			Intake		Output			Intake minus output
			in diet	in excess R.B.C. injected	in plasma	in urine	in feces	
		kg.	gm.	gm.	gm.	gm.	gm.	gm.
	Kennel	11.6						
1	Fasting	10.3	0.0	−0.3	4.8			
2	Liver basal	10.4	13.4	−0.1	4.7			
3	Liver basal	10.7	13.4	2.8	3.8			
4	Liver basal	10.7	13.4	0.8	3.6			
5	Liver basal (anaphylactic shock)	10.6	11.5	2.6	3.2			
6	Liver basal	10.8	13.4	2.4	4.8	6.7	2.9	+1.4
7	Liver basal	10.9	13.4	1.4	4.5	8.3	1.2	+0.8
8	Liver basal	11.1	13.4	−2.0	3.9	6.3	3.8	−2.6
9	Liver basal	11.3	13.4	1.0	4.0	8.3	3.0	−0.9
10	Liver basal	11.4	13.4	0.7	3.2	7.7	1.4	+1.8
11	Liver basal	11.6	13.4	−0.2	4.0	7.4	1.9	−0.1
12	Liver basal	11.7	11.2	−1.9	3.3	6.6	1.7	−2.3
13	Liver basal	11.8	11.2	−1.1	2.8	6.8	2.0	−1.5
14	Liver basal + gelatin, 69 gm.	12.0	21.4	−0.8	2.8	15.5	2.7	−0.4
15	Liver basal	12.0	11.2	−0.6	3.1	8.3	2.8	−3.6
16	Liver basal + gelatin, 70 gm. + tryptophane, 2.8 gm.	12.3	22.2	2.6	3.1	15.8	1.9	+4.0
17	Liver basal	12.5	11.2	2.5	3.5	9.1	2.1	−1.0
18	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + tryptophane, 2.8 gm.	12.5	22.6	7.8	4.2	13.3	2.8	+10.1
19	Liver basal	12.7	11.2	3.3	5.0	10.0	3.4	−3.9
20	Liver basal	13.1	11.2	4.6	3.8	8.7	2.6	+0.7
21	Liver basal	12.7	11.2	1.6	3.2	9.8	2.4	−2.6
22†	Liver basal + gelatin, 70 gm. + cystine, 4.2 gm. + phenylalanine, 4.2 gm.	12.4	22.6	2.3	3.7	19.1	3.1	−1.0
23	Liver basal	—	11.2	1.4	3.8	8.8	2.3	−2.3
	Totals, periods 6 to 23..............		258.8	25.0	66.7	176.5	44.0	−3.4

† See footnote to period 22, Table 3. The figures for nitrogen balance are 7/6 of those obtained.

plasma protein—a potency ratio of 4 and an efficiency percentage of 27. Dog 36-196 required 6 gm. of diet protein to produce 1 gm. plasma protein under identical circumstances (Table 4). This dog (37-6) during basal periods put out an average of 7.9 gm. urinary nitrogen while dog 36-196 eliminated 8.8 gm. urinary nitrogen per week under identical conditions. Dog 37-6 which conserved its proteins to better advantage was a quiet, calm, inactive dog, but dog 36-196 was very active at all times. It is possible that this difference in activity was a factor but we do not wish to stress the point. We have no further comments to make relative to such individual differences except that they exist. It has been noted that some dogs can produce more new hemoglobin during experimental anemia than other dogs under identical conditions (14) and it is a commonplace that some dogs can run faster than others. Undoubtedly many unknown factors are involved in these individual differences.

The gelatin amino acid supplements gave responses similar to those recorded in Tables 1 and 2. Gelatin plus cystine and tryptophane gave a large production of plasma protein (24 gm.) in period 18, Table 3. It is significant that the albumin/globulin ratio showed a sharp rise to 1.16 indicating presumably a sharp increase in albumin production. This is a familiar response (7) to the feeding of proteins favorable for plasma protein production (usually meat proteins). In contrast, this dog with gelatin feeding alone showed no increased output of plasma protein (period 14, Table 3) and when the gelatin was supplemented with tryptophane the increase was scarcely recognizable (period 16, Table 3).

Phenylalanine obviously cannot effectively replace tyrosine in the potent cystine, tyrosine, gelatin mixture (Table 1, period 23). The phenylalanine, cystine, gelatin mixture showed an increase of 7.5 gm. plasma protein (period 22, Table 3) or about one-third the amount to be expected from the tyrosine, cystine, gelatin supplement. The urinary nitrogen figures are completely out of line with all other observations, and we have no adequate explanation.

The urinary nitrogen figures in periods 14, 16, and 18, Table 3-a, are in harmony with those recorded above (Tables 1-a and 2-a). With gelatin alone or gelatin plus tryptophane the urinary nitrogen is higher than is observed with the cystine, tryptophane, gelatin mixture, favorable for plasma protein production.

Clinical Experimental History.—Dog 37-6 (Tables 3 and 3-*a*). An adult female mongrel pointer, weighing 11.6 kg., was fasted for one week. For the succeeding 10 weeks it was fed daily a basal ration consisting of raw pork liver, 60 gm.; cane sugar, 120 gm.; lard, 15 gm.; cod liver oil, 15 gm.; salt mixture, 2 gm.; bone ash, 10 gm. In the 5th week horse serum antigen given intravenously to the previously sensitized animal, as an aside to the main experiment, produced a severe characteristic shock. Within 24 hours the dog had recovered for the most part and appeared entirely normal within 3 days. Beginning with period 12 the amount of liver in the basal diet was reduced to 50 gm. daily and the cane sugar was increased to 124 gm. During the periods in which gelatin supplement was given the quantity of cane sugar in the diet was reduced to 115 gm. The appetite for the basal diet lagged by the 21st period and during the 22nd week a total of exactly one day's diet was not eaten. Since a considerable gain in weight had occurred the lard was deleted from the diet beginning with the last 2 days of the 21st period; and the carbohydrate was reduced to cane sugar, 60 gm., cornstarch 30 gm. during the 23rd period. The experiment was discontinued at the close of this period.

DISCUSSION

Plasma protein is not to be looked upon as static material whose sole function is related to the osmotic balance of circulating and tissue fluids. This plasma protein material has a *turn over* of unknown value but the body can be kept in nitrogen equilibrium on a non-protein diet plus adequate plasma given intravenously (2). In other words the plasma protein can replace the body protein wear and tear and in this sense is active in nutrition under such conditions. It is obvious therefore that information as to plasma protein production is of significance relating to body protein exchange and metabolism (12).

In a comprehensive review (11) Rose shows that for *growth* the amino acids phenylalanine and methionine are indispensable but tyrosine and cystine are dispensable. The experiments above, summarized in Table 4, indicate that for *plasma protein building* methionine cannot take the place of cystine as a supplement to gelatin and phenylalanine cannot replace tyrosine in the same type of reaction.

It would seem that the urge to produce plasma protein in these depleted dogs was maximal and the dog uses the liver protein in the basal diet quite efficiently—4 to 6 gm. of liver protein being required to produce 1 gm. of plasma protein. Gelatin supplemented with cystine and tyrosine is used as efficiently as beef serum itself to build new plasma protein, 3 to 4 gm. of gelatin protein being needed to

produce 1 gm. of plasma protein. When gelatin is supplemented with cystine and phenylalanine or methionine and tyrosine, it is utilized poorly and it requires about 9 gm. of gelatin protein to pro-

TABLE 4
Summary of Experiments on the Influence of Certain Amino Acids in Plasma Protein Formation

Reference	Efficiency in plasma protein formation: Protein output per cent of protein intake				
Dog No.	Tables 1, 2	Table 3	(5)	(6)	(9)
	36-196	37-6	33-11	34-152	32-130
Basal Diets					
Liver					
Kidney					
Potato-bran	17	27	22		
Supplements					
Beef serum (best protein yet tested)				22	
Gelatin + cystine + tyrosine + tryptophane					24
Gelatin + cystine + tyrosine	34				
Gelatin + cystine + tryptophane	39, 25				38
Gelatin + cystine	32				
Gelatin + tyrosine	10	41			
Gelatin + tryptophane	5				
Gelatin + tyrosine + tryptophane	3±	3			
Gelatin + cystine + phenylalanine	1		33		
Gelatin + methionine + tyrosine	6	13			
Gelatin + methionine	12				
Gelatin	5				
Tryptophane		2±			
Lysine			9		
Cystine + glycine + glutamic acid	0*	0*		0*	
Histidine + lysine + arginine				50*	
				26*	

* These figures represent the percentage increase in the basal plasma protein output induced by the amino acid supplement.

duce 1 gm. of new plasma protein (Table 4). Gelatin alone does not contribute to the building of new plasma protein.

Valine should be mentioned even if we have no experiments with this amino acid to report at this time. Valine is listed by Rose (11) as indispensable for growth. What its significance may be relating

to plasma protein production we cannot say but certainly it does not appear to be indispensable as a gelatin supplement. Gelatin supplemented with cystine and tyrosine is as potent as beef serum in the building of new plasma protein in these depleted dogs and it is hard to imagine that valine would add to the potency of this gelatin mixture. However, it must be put to the biological test.

SUMMARY

When blood plasma proteins are depleted by bleeding with return of the washed red blood cells (plasmapheresis) it is possible to bring dogs to a steady state of hypoproteinemia and a uniform plasma protein production on a basal low protein diet. These dogs are clinically normal. By the introduction of variables into their standardized existence insight into the formation of plasma proteins can be obtained.

The liver basal diet maintains health in such hypoproteinemic dogs during periods as long as a year. 17 to 27 per cent of its protein content (entirely liver protein) is presumably converted into plasma protein.

Gelatin alone added to the liver basal diet causes very little if any extra plasma protein production.

The addition to gelatin of cystine, or tyrosine, or tryptophane, or of both tyrosine and tryptophane has little or no effect on its potency for plasma protein production.

When gelatin is supplemented by cystine and either tryptophane or tyrosine, 25 to 40 per cent of the protein content of the combination is converted into plasma protein—an efficiency equaling that of any protein hitherto tested.

Preliminary experiments indicate that methionine cannot substitute for cystine nor can phenylalanine substitute for tyrosine in the efficient combination of gelatin plus cystine plus tyrosine.

Laked red blood cells given by vein afford little or no material for plasma protein formation.

When the *reserve stores* of plasma protein building material are exhausted the dog can form little if any plasma protein during protein-free diet periods.

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RADIOACTIVE IRON AND ITS METABOLISM IN ANEMIA*

Its Absorption, Transportation, and Utilization

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With the production of radioactive isotopes by the physicists and the concentration of naturally occurring isotopes by the chemists, the grateful physiologists have been presented with what may prove to be the "Rosetta Stone" for the understanding and study of body metabolism. The radioactive isotopes are "marked" elements which behave precisely like their inactive replicas in the physiology of the body but can be readily recognized as distinct entities wherever found. The radioactive isotope of iron used fortunately has a long life (half life 47 days) which covers ample time for prolonged study of iron metabolism and gives assurance to the student that this iron found in tissues, bones, or fluids is the iron introduced and not some other iron coming from body storage depots, hemolysis, or red cell wastage.

The literature on iron metabolism is enormous but the net results are disappointing to say the least. Every hypothetical possibility has been championed by students but very few points have been settled to the satisfaction of all workers. One can scarcely suggest a single possibility without finding that it has been more or less vigorously supported by physiologists or physicians in the past. We feel that radioactive iron will be a means of settling many of these disputes but it is impossible at this time to review all these interesting hypotheses.

The metabolism of iron in the body at first sight appears to be simple and capable of accurate study but this illusion is promptly

dispelled when the student deals with body secretions, tissues, and bones. Iron exists in many combinations and in many places in the body—red cell hemoglobin, muscle hemoglobin, liver, spleen, and marrow tissue, to name the most important. Moreover the picture is complicated further because in nearly all tissues iron may be present in two or more entirely different forms. In the spleen for example, there is parenchyma iron which is essential to the very life of the tissue; iron in the contained blood; and storage iron, a labile reserve which may be brought into play for any of the body needs. In fact the blood iron itself consists of several entities. It is quite apparent that analysis of iron by chemical methods alone could give but little information concerning the meaning of changes in total splenic iron.

The body can mobilize its iron under certain conditions. We have little accurate knowledge as to the length of its stay in various depots. Method difficulties are very real and the magnitude of method errors is not understood by many workers in this field. The presence of calcium and phosphorus introduces serious error in the iron analyses of the feces and the bone marrow, the very places where accurate analyses are imperative.

There are recognized fractions of iron (other than in hemoglobin combination) in the plasma and red cells but the quantities of iron are very small and their determination difficult due to the fact that traces of hemolysis may confuse the picture. It is very difficult to be sure in spite of every precaution that the blood has been drawn without causing a trace of hemolysis. Study of *iron transport* is obviously of major importance and it can scarcely be approached without the aid of radioactive iron.

Iron absorption has been assumed rather than demonstrated because of method difficulties. Some years ago we (6) brought evidence to show that in the iron depleted anemic dog the iron is whisked rapidly from the intestine through the liver and marrow into the red cells. The rapidity of this movement of iron surprised us but the evidence accumulating from the use of the radioactive iron indicates that the "turnover" of iron is even more rapid than was then suspected.

When radioactive iron is fed one can readily distingui...

newly acquired iron and that present in the body even though the inert iron exceeds the radioactive iron by many hundred-fold. This contrasts with iron metabolism studies using inert iron where in many cases significant results depend on small differences between analytical iron values not too certain in themselves.

The *excretion* of iron has been the subject of debate by physiologists for decades and until very recently no voice was raised to question the function of the large intestine which had been accepted as the site for iron elimination. Radioactive iron should enable the student to settle this dispute beyond any reasonable doubt. Furthermore the iron metabolism of the muscle hemoglobin has never even aroused speculation but this question now can be approached with hope of ultimate success.

Absorption of iron in disease is probably abnormal and these questions call for the use of radioactive iron in the clinic.

Methods

Radioactive iron is formed by the bombardment of the Fe^{58} iron isotope with deuterons, the reaction being $Fe^{58} + H^2 \longrightarrow Fe^{59} + p$. It decays with the emission of free electrons or β-rays, $Fe^{59} \longrightarrow Co^{59} + e$. The half life of radioactive iron is 47 days. The amount of radio-iron present in a sample is determined by a measurement of its β-ray activity using a Geiger-Müller counter. The experimental methods for doing this are basically those described by Bale, Haven, and LeFevre (1)

Briefly the method is as follows: A 2 cc. aliquot of the solution on which an activity determination is to be made or the whole sample made up to 2 cc. is placed in a glass cup the inside diameter of which is about 1 mm. greater than the outside diameter of the counter chamber, which is mounted as an inverted plunger. A rack and pinion device (adapted from a colorimeter) brings the cup up around the counter end to a point where the liquid forms a $\frac{1}{2}$ mm. thick film surrounding the sensitive area of the counter. Such geometric conditions allow many β-rays to enter the counter tube producing a high sensitivity. By using equal volumes of solution and by raising the rack and pinion to the same point on a scale, the same geometric conditions are obtained for each sample and the resulting counts per minute are proportional to the radioactivity of the sample.

A nearly saturated solution of potassium acetate is a convenient preparation of constant radioactivity. It is advisable to check the sensitivity of the counter at frequent intervals using this standard. Background counts (around three or four a minute for our counters) are determined on distilled water, or on acid blanks approximating the composition of the solvent for the sample being measured.

Two characteristics of radio-iron make its quantitative determination difficult. The preparations so far available have been extremely weak, compared for example with radioactive phosphorus or sodium preparations that are available. This means that in many aliquots on which activity determinations are to be made, activities are close to the minimum measurable, and experimental errors are likely to be large.

Also, the β-rays of radio-iron are of low energy and consequently easily absorbed (7). This makes necessary careful extractions of iron and in many cases use of relatively large amounts of tissue and its purification from foreign substances that would otherwise absorb the β-radiation and give too low apparent radioactive intensities. It is also necessary to make corrections in iron solutions that are not extremely dilute for its self-absorption of β-radiation. All of these factors tend to make the apparent recoveries of radio-iron in our biological experiments less than 100 per cent. This means that results in which recoveries are low should probably not, in general, be interpreted as showing a deposition of iron in unknown depots, but rather that our assay methods have still to be improved to be completely quantitative.

As has been stated above, it was found necessary to prepare the material for counting so that a relatively pure solution of iron salt was being dealt with. This minimized the absorption of β-rays by foreign materials. In the instance of plasma and red blood cell samples, as well as some of the viscera containing little calcium and phosphorus, the following procedure was employed:

The material in a conveniently sized Kjeldahl flask was wet ashed in a volume of concentrated sulfuric acid varying from 7 to 80 cc. depending upon the amount of organic material present. Perchloric acid was added as needed after charring occurred and in the event of excessive frothing caprylic alcohol was added. When the solution was clear and colorless it was cooled, diluted with an equal volume of distilled water, and cooled again. With phenol red as an indicator, the solution was brought to near neutral with 40 per cent sodium hydroxide and transferred to 100 ml. centrifuge tubes. Neutralization was completed and the precipitate centrifuged at about 2500 R.P.M. for 15 to 20 minutes. The supernatant was discarded and the precipitate dissolved in a little concentrated hydrochloric acid. If the resultant solution was not clear about ½ to 1 gm. of ammonium chloride was added and after diluting with water and heating to dissolve the salt more indicator was added and the iron reprecipitated. After centrifuging as before, the solution was again dissolved in acid, and if clear, was made to 2 ml. and transferred to the cup for counting. If moderate amounts of calcium were present, 4 or 5 repetitions of ammonium chloride additions with subsequent precipitation often succeeded in getting rid of it. In the treatment of material high in calcium and phosphorus such as bone, food, or feces, the material was precipitated several times and the mixture of the hydroxide and extraneous salts was treated with an excess of concentrated hydrochloric acid. After evaporation to dryness, the material was transferred to a continuous extractor similar to that described by Griffith (3) by means of repeated washings with concentrated,

hydrochloric acid. The mass was extracted with ethyl ether until the acid phase showed a negative test for iron using thiocyanate. It was often necessary to add ammonium persulfate to take care of reduction occurring during extraction (presumably due to impurities in the ether) since ferrous iron is not readily extracted. The ether extract was finally washed into beakers with dilute acid and concentrated over a steam bath, and finally made up to 2 ml. and counted.

Some samples contained very small amounts of radioactive iron where the total count was very near the background and due to inefficiency of the counter tube employed, quantitative results were impossible. This was especially true if weak samples of the isotope had been fed. In such cases the self-absorptive factor as well as absorption by solvent could be partially avoided by resort to electroplating of the iron onto tin foil cylinders made to fit snugly around the counter tube. Such a procedure resulted in obtaining 6 to 8 times the number of counts as were obtained on the same material in solution. This method will be described fully in a forthcoming publication.

Routine care of these animals including methods of bleeding; determination of plasma and blood volume; and hemoglobin, as well as the composition of the diet low in iron, have been described in detail (6, 11). Blood volumes were done weekly by the brilliant vital red dye procedure. Hemoglobin was determined by the acid hematin procedure (13.8 gm. per 100 cc. taken as 100 per cent). Viviperfusion is also described (10) but in brief it consists of bleeding the dog under ether anesthesia at the same rate at which a modified Locke's solution is given intravenously. This leaves the tissues practically free of red cells excepting the spleen and bone marrow.

Values determined from single samples of plasma or red cells are expressed as total amounts in circulation by applying plasma and blood volume corrections respectively. Marrow samples were corrected roughly to indicate total body marrow tissue according to figures for marrow volume given by Fairman and Whipple (2).

EXPERIMENTAL OBSERVATIONS

Over a period of years investigations have been carried out in the Rochester laboratory relative to the absorption, storage, and utilization of iron in dogs which had been previously depleted of all reserve iron stores by continued anemia and subsistence on diets low in iron (6, 8). The limitations of these procedures have been outlined in detail (4). It seemed advisable to repeat some of the typical experiments using iron containing the radioactive isotope to test the accuracy of this method under actual laboratory conditions.

Dog H-9, a young female terrier, weighing 4.5 kilos (Table 1) was placed on a diet low in iron, consisting chiefly of white bread and canned salmon (6). It was

made anemic by bleeding and the anemia was maintained for 3 weeks. Radio-iron was fed (in the form of $Fe_2(SO_4)_3$) at a level of about 55 mg. daily (showing 192 counts per minute) for 4 days. At the time of the first feeding the blood hemoglobin level was 39 per cent (13.8 gm. hemoglobin per 100 cc. = 100 per cent). 20 hours after the last feeding the animal was subjected to viviperfusion to render the viscera blood free (10) and the perfusate was collected in several fractions. The final hematocrit at the end of this procedure was 0.4 per cent in contrast to 17 per cent at the beginning.

TABLE 1

Radioactive Iron Content of Tissues

Tissue or Organ Content = Per Cent of Total Amount Fed

Dog No.	Anemic						Normal		
	H-9	H-8	37-116	37-227	37-204	37-202	37-77	37-144	37-214
Number of feedings	4	2	1	1	1	1	18	5	1
Blood volume	330	350	500	370	440	770	480	630	700
Plasma volume	260	260	390	250	330	620	240	360	400
Iron fed in mg.	220	66	130	84	300	115	650	103	60
Counts per minute as fed	770	464	5,730	21,500	6,590	13,000	600	2,120	14,240
Hb. level per cent when fed	39	62	53	68	61	56	178	138	114
Hours after last feeding	20	20	23	4	75	11	26	6	84
Radio-iron found									
Liver	0.4	0.4	0.5	—	—	—	0.2	0.03	0.02 / 7
Spleen	0.0	0.0	0.1	—	—	—	0.0	0.02	0.01
Marrow	0.2±	3.0±	2.0±	—	—	—	0.0	0.03±	—
Plasma	0.0	0.3	0.1	0.7 / 0.1 / 0.8 / 0.2			0.0	0.01)	
Red cells	8.7	9.0	1.4	0.9 / 4.6 / 0.0 / 0.4		3.5	0.2	0.04 / 0.06}	
Total radio-iron	9.3±	12.7±	4.1±	1.6 / 4.7 / 0.8 / 0.6		3.7	0.24	0.15±	0.05 / 0.08

Radioactivity measurements on the ashed viscera and blood show (see Table 1) that a total of 9.3 per cent of the amount fed was absorbed during the feeding periods. Most of this (8.7 per cent) was already in the red blood cells at the end of 5 days. The liver and bone marrow (estimated total) contain the remainder (0.6 per cent). The colon and feces accounted for about 63 per cent of the amount fed. The remainder (26 per cent) represents the inefficiency of recovery of material at this time.

. Dog H-8, a female mongrel fox terrier puppy, aged about 6 months, and weighing 4.2 kilos, was made anemic by bleeding and placed on a white bread-salmon diet low in iron. The anemia was continued for 9 weeks to remove all iron reserves. Iron containing the radioactive isotope was fed with the diet in the form of $Fe_2(SO_4)_3$, on 2 successive days at a level of 33 mg. per day. The count of each dose was 232 per minute. 20 hours following the last feeding, viviperfusion was carried out.

Analysis of the viscera of this animal for radioactivity showed 0.4 per cent of the amount fed in the liver (Table 1), approximately 3 per cent in the marrow, and 9 per cent in the red blood cells. The plasma showed a count of 0.3 per cent corresponding to 0.2 mg. of iron. About 70 per cent was recovered from the colon and feces.

Dog 37-116, a female mongrel adult, weighing 6 kilos, was made anemic by bleeding and placed on the usual diet low in iron. The anemia was continued for 5 weeks to deplete the reserve. For a month preceding administration of radioactive iron there had been a leucocytosis which persisted to the date of perfusion. The animal was given a single dose of radio-iron as $Fe_2(SO_4)_3$ mixed with the diet, consisting of 130 mg. of iron which counted 5,730 per minute. The animal had to be coaxed to eat and spent one-half hour eating most of the food. The remainder was given by gavage. Blood samples were taken 3 and 5 hours after start of feeding and showed hematocrits of 20.9 and 19.8 per cent, respectively. Viviperfusion was carried out 23 hours following feeding (see Tables 1 and 2). The hematocrit at the beginning was 19.9 per cent and at the end 1.1 per cent.

At autopsy the organs appeared well perfused. Heart, lungs, kidneys, liver, and spleen appeared to be normal, in gross. There was gross and histological evidence of a moderate grade of chronic endometritis. The other viscera were ashed *in toto* for analysis.

The absorption of iron is much less in this experiment than in the two preceding—a total found of only 4 per cent. This is due to the feeding of a large single dose of iron which is known to lessen the percentage absorbed (optimum percentage absorption follows 20 to 40 mg. iron per day). Absorption of 30 per cent may be found in anemic dogs (8) with doses of 30 to 40 mg. iron daily, but absorption of only 5 per cent may be found with doses of 400 mg. iron. The red cells here contain nearly one-third of the absorbed iron even after 23 hours following rather reluctant and prolonged ingestion of the iron.

RADIOACTIVE IRON AND ITS METABOLISM IN ANEMIA

TABLE 2
Radioactive Iron as Found in Body
Figures in Table = Per Cent of Iron Given

Dog 37-116. Female adult mongrel, 6 kilos. Fed single dose of 130 mg. of radioactive iron (counting 5,730 per minute).

	Plasma*	Red cells*
Sample at 3 hrs.	1.06	0.23
Sample at 5 hrs.	1.36	0.24

†Fractions from viviperfusion 23 hrs. after feeding	Plasma total	Red cell ½ total	Red cell protein†	Red cell protein-free filtrate†
Fraction 1 (170 cc.)	0.063	0.305	0.36	0.013
Fraction 2 (220 cc.)	0.028	0.26	0.24	—
Fraction 3 (170 cc.)	0.016	0.08	0.10	0.005
Fraction 4 (175 cc.)	0.005	0.03	—	—
Total 735 cc.	0.11	0.68	0.70	0.02

Liver (153 gm.)....0.53%
Spleen (16 gm.)....0.12%
Marrow..........2.0±%

Whole blood.......1.41%
Stomach and small intestine.........0.46%
Colon............52.0%

* The 3 and 5 hour plasma samples were corrected to represent the iron in the total circulating plasma, and the red cell samples for total circulating cells, using plasma and blood volumes respectively as determined beforehand.

† Red cell fractions of the perfusate were divided into 2 equal parts. One-half was subjected to ashing and counting directly. The other was laked in each case and the proteins precipitated with trichloracetic acid and the precipitates and filtrates ashed separately.

Dog 37-227, an adult male poodle, weighing 4.5 kilos, was made anemic by bleeding and kept anemic for 5 weeks. During this time the diet was of hospital table scraps. The low iron diet was then begun and 4 days later a sample of iron containing the radioactive isotope was given in the form of FeCl$_3$. 84 mg. were given, which counted 21,500 per minute (see Chart A). Samples were taken at periods after feeding of 1, 2, 4, 12, and 24 hours. In each case the plasma and cells were separated by centrifugalization. One-half of the red cells were laked in distilled water and run into 40 cc. of 10 per cent trichloracetic acid. The plasma samples were treated similarly. With frequent shakings, all samples were allowed to stand for 15 minutes. After filtration the precipitates and filtrates were each ashed and prepared for counting.

It was found that nearly 80 per cent of the iron in the plasma was either not in protein combination in so far as it appeared in the trichloracetic acid filtrates, or that the latter acid has split it from loose combination. The radio-iron of the red cells was almost entirely in the fraction precipitated by the trichloracetic acid, only traces occurring in the filtrates of the precipitated laked cells.
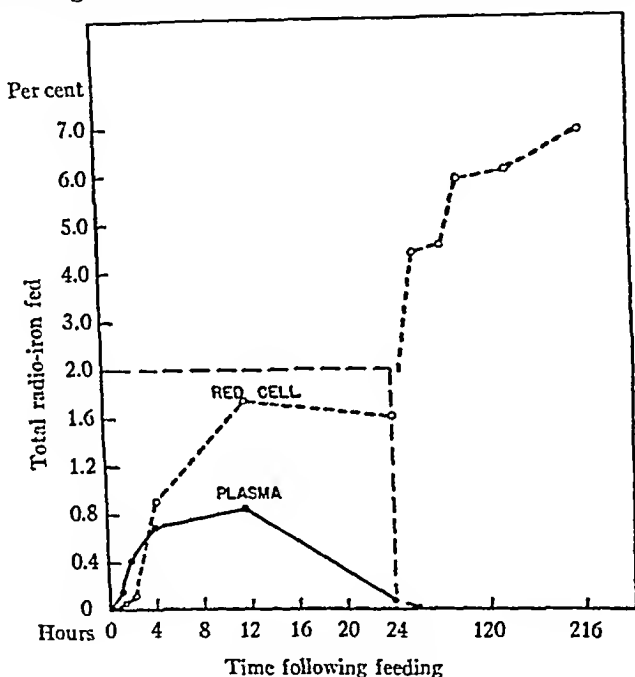
CHART A. Plasma and red blood cell radio-iron. Single feeding experiment. Dog 37-227.

The result can be seen in Chart A. This animal was studied for a long period and gradual depletion of the radio-iron in circulation was effected.

Dog 37-204, a male adult poodle, weighing 4.5 kilos, was made anemic and placed on a diet low in iron. It might be noted that this animal had practically no reserve iron stores. After an anemia period of 3 weeks, 300 mg. of iron containing the radioactive isotope was mixed in the diet in the form of FeCl₃. This amount counted 6,590 per minute. About 80 per cent of the diet was eaten immediately and the rest later in the day. A blood sample was taken 2 hours following feeding and others at 11 and 26 hours.

No radio-iron was detectable in the 2 hour samples of plasma or red cells of this animal. None was detectable in the 11 hour sample of red cells but the total blood plasma contained 0.8 per cent of the amount fed (Table 1). The 26 hour sample of plasma showed 0.2 per cent, while 0.4 per cent of the amount fed was present in the circulating red blood cells. 6 days after the feeding there was 3.8 per cent in the circulating red cells.

The feces collected during a 9 day period following the feeding showed 63 per cent of the amount of radio-iron fed. The low absorption of 3.8 per cent was probably referable to the high level at which the iron was fed (300 mg.).

Dog 37-202, an adult female mongrel spitz, weighing 10 kilos, was placed on a diet low in iron and made anemic by bleeding. After 4 months on this régime a sample of radioactive iron was given in a part of the diet, representing 115 mg. and counting 13,000 per minute. Samples were taken at 1, 4, 6, 8, 10, 12, 15, 19, and 24 hours and daily thereafter for several days.

Absorption in this animal as indicated by both the plasma iron determinations shortly after feeding and by the ultimate amount appearing in the red cells was quite low, amounting to only about 3.7 per cent of the iron fed. However it might be stated that the peak of absorption as shown by the plasma radio-iron occurred between the 5th and 7th hours reaching a value at the end of the 6th hour of about 4 times that in the preceding or following hour. Thus in Chart A the curve may not show accurately the changes in plasma radio-iron. More complete data are being compiled concerning this point and will be presented in detail. Practically all of the iron (95 to 98 per cent) in the plasma was in the filtrate following precipitation of the proteins with trichloracetic acid. A duplicate sample of plasma taken 12 hours after feeding was dialyzed in a cellophane membrane against running tap water for 24 hours. The resultant material was ashed and showed no activity.

Control Non-Anemic or Plethoric Dogs.—Dog 37-77, an adult female bull terrier, of 5 kilos weight, was placed on a diet of hospital table scraps, supplemented by 400 mg. of iron in the form of ferric citrate, daily for 12 days. In addition, a series of whole blood transfusions were given over a period of a week, during which 53 gm. of hemoglobin, equivalent to 178 mg. of iron, were injected

by vein.   As the animal was normal at the start, it was felt that there was no doubt that the iron stores were abundant.   Before the transfusions, the hematocrit was 48 per cent with hemoglobin level of 120 per cent.   At the end of the whole blood administration the hematocrit was 63 per cent and the hemoglobin level was 178 per cent.   One transfusion of whole blood (equivalent to 5.4 gm. hemoglobin) followed this determination.

Radio-iron, as recovered from other experiments, was fed nearly every day over a period of 5 weeks.   The total iron given during this time approximated 650 mg. and counted about 600 per minute.   84 hours after the last feeding, the animal was perfused and the organs, all of which appeared normal in gross, were ashed for counting.

The colon contained 0.9 per cent of the total amount of iron fed, the remainder of the gastro-intestinal tract only 0.03 per cent.   None was found in the vertebral bodies, plasma, or spleen.   The liver contained 0.2 per cent and the combined red cells 0.04 per cent of the amount fed.   It is apparent that very little iron was absorbed (see Table 1).

Dog 37-144, a female adult beagle mongrel, weighing 8 kilos, was given several transfusions of whole blood (21 gm. of hemoglobin equivalent to 70 mg. of iron). White bread-salmon diet, with a supplement of 400 mg. of iron daily was given for 5 weeks.   During this time occasional injections of colloidal Fe(OH)$_3$ were given by vein, 64 mg. at a time.   The total amount of colloidal Fe injected in this way was 394 mg.   The animal originally had an hematocrit of 50 per cent and a hemoglobin level of 138 per cent, so the iron storage depots could be assumed to be full.   Radio-iron (20 mg.) recovered from another experiment was fed with the diet and 2 days later 40 mg. of the same material were given.   On each of the next 2 days, 21 mg. of a new sample were given in the diet.   The total radio-iron intake was 103 mg. and counted 2,120 per minute.   23 hours later the animal was perfused.

The viscera of this animal appeared normal and were ashed for counting.   The activity can be seen in Table 1.   The stomach contained 0.11 per cent of the amount fed, the combined jejunum and ileum 0.13 per cent, and the colon, with contained feces, 82 per cent. It is to be noted that less than 0.2 per cent was absorbed as determined by analysis of blood and tissues.

Dog 37-214, an adult female mongrel spitz, weighed 7 kilos.   This animal was vaccinated for distemper but did not receive the virus because of some coughing. There was no nasal secretion.   A single transfusion of whole blood (8 gm. of hemoglobin equivalent to 27 mg. of iron) was given.   Eight injections of 64 mg.

each of iron as colloidal Fe(OH)$_3$[1] were given at various times over a period of 10 weeks to insure excess iron storage.   The initial hematocrit was 37 per cent with a hemoglobin level of 105 per cent.   At the end of injections the hematocrit was 42 per cent and the hemoglobin 114 per cent.

A sample of radio-iron recovered from a previous experiment was fed, which contained about 60 mg. of iron and counted 14,240 per minute.   Perfusion followed feeding by 7 hours.

The very small amounts of radioactive iron found in the viscera of this animal can be seen in Table 1.   The marrow sample was lost. The whole gastro-intestinal tract contained 84 per cent of the amount fed.

It is important to compare this dog 37-214 with the anemic dog 37-202.   Both dogs were given about the same amount of radioactive iron as measured by the counts per minute.   The anemic dog received 115 mg. Fe in contrast to the control non-anemic dog which received 60 mg. Fe.   This would favor more absorption (in per cent) in the control.   Yet the anemic dog showed 3.7 per cent in the blood in contrast to 0.05 in the non-anemic dog—an impressive ratio of over 70 to 1 for the absorption in anemia.

DISCUSSION

The apparent ability of the dog to discriminate physiologically as concerns the amount of iron absorbed is of immediate interest (5). When there is a distinct need for the element (anemia) a fair quantity will pass from the gastro-intestinal tract into the blood stream. When the body reserves of iron are ample, very little is assimilated (Table 1).   The mechanism for this reaction is not known.   That it may be dependent upon a concentration gradient existing between the gastro-intestinal contents and the mesenteric blood is very doubtful since the iron in the blood (in a form other than hemoglobin) is present in very small amount in any case as compared with the amount in the gastro-intestinal tract itself.

It is interesting to speculate concerning these data however.   If a normal animal absorbs iron only in proportion to the need and the anemic animal utilizes very efficiently (4, 6) what is absorbed then the

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[1] We are indebted to Dr. David Loeser of the Loeser Laboratories, New York, for the colloidal iron used in these experiments.

expected excretion requirement in either case would be very small. This is pretty much what is found by means of bile fistula dogs (to be published later). The same conclusion has been reached by Widdowson and McCance (12) who recently carried out a series of iron balance studies on human subjects. They found that iron injected in normal individuals did not result in an appreciable iron elimination and concluded that the excretory power of the gastro-intestinal tract was very limited. In a patient with an ileostomy stoma and isolated colon, Welch, Wakefield, and Adams (9) showed that feeding of large amounts of iron by mouth did not result in its excretion by the colon.

Some iron is excreted in the bile and under abnormal conditions (unpublished data) it may be increased several fold, but it does not account for the total iron excretion of the gastro-intestinal tract under normal conditions and probably for less than one-third of the total.

One may choose to believe that the *iron absorption* is largely a concern of the small intestine and furthermore that the *mucosa* is the tissue responsible for its acceptance or rejection. It may be possible to show that the epithelium of the mucosa is conditioned by the anemic state of the circulating blood so that absorption of iron takes place. At any rate the curve of iron absorption by the anemic dog indicates that the peak absorption (4 to 8 hours after feeding) takes place when the food materials are largely in the *small intestine*. At the end of 18 to 24 hours the radioactive iron is practically all in the colon and no significant absorption of iron is demonstrable. It would seem that the colon is not concerned with iron absorption.

That the plasma is the site of *transportation of iron* from the intestinal tract to the point or points at which it is further utilized is indicated by the above data (Tables 1 and 2). Further evidence along this line will be presented later but like many other materials the absorption, as indicated by plasma iron changes, increases shortly after feeding to a peak and drops off quite rapidly (Chart A). Absorption from a given dose of iron probably is complete in the dog at the end of 18 hours.

The rapidity with which the radio-iron appears in the red blood cell is worthy of comment at this time. If an anemic dog is fed neutral iron the earliest time in which it appears in the blood stream as shown by the red cell surge into the circulation, is about 3 to 5 days. How-

ever we see in dogs 37-227, 37-204, 37-116, and 37-202 (Tables 1 and 2) that the radio-iron has found its way into the red cells much sooner; in fact appreciable amounts are demonstrable in a few hours.   Further studies of the transfer of iron to the red cells and the form in which it occurs there are being made.

As has been pointed out (6) the spleen and marrow do not lend themselves to complete removal of blood by viviperfusion. About one-third of the iron found on analysis of a perfused spleen from an anemic depleted dog is due to the presence of hemoglobin in the red cells contained in the sinusoids (6).   The question might arise concerning the radio-iron found in the spleen of dog 37-116; whether it was present as true storage iron or represented radio-iron in the contained blood. Calculation shows that less than 10 per cent of the radioactive isotope present (0.12 per cent of the total amount fed) could be accounted for as hemoglobin iron.   The remainder of the radioactive iron in the spleen presumably was in the reticulo-endothelial cells as a reserve store of readily mobilizable iron.

Discrepancies in iron balance studies in human subjects have sometimes been explained as due possibly to *adsorption* of iron to the mucosa of the gastro-intestinal tract leading to a false impression of positive balance.   It is of interest to note that in both dogs 37-116 and 37-144 where viviperfusion followed the last feeding of radio-iron by 23 hours, practically all of the iron had been swept through the upper gastrointestinal tract and had either been eliminated or was in the colon. The stomach and small intestine of 37-116 contained only 0.46 per cent of the total isotope fed whereas the colon contained 52 per cent. The upper gastro-intestinal tract of 37-144 contained 0.24 per cent of the amount of radio-iron fed while the colon and contained feces contained 82 per cent.   The material fed in each case consisted of iron in the ferric form which would be expected to show adsorption (if present) to a greater extent than ferrous iron compounds.

<center>SUMMARY</center>

Artificially produced radioactive iron is an extremely sensitive agent for use in following iron in the course of its changes in body metabolism, lending itself to studies of absorption, transport, exchange, mobilization, and excretion.

The need of the body for iron in some manner determines the absorption of this element.   In the normal dog when there is no need for the element, it is absorbed in negligible amounts.   In the anemic animal iron is quite promptly assimilated.

The plasma is clearly the means of transport of iron from the gastro-intestinal tract to its point of mobilization for fabrication into hemoglobin.

The speed of absorption and transfer of iron to the red cell is spectacular.   The importance of the liver and bone marrow in iron metabolism is confirmed.

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ANTIGEN-ANTIBODY REACTIONS BETWEEN LAYERS ADSORBED ON BUILT UP STEARATE FILMS

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Langmuir and Schaefer (1, 2) have developed a method of conditioning barium stearate films for the adsorption from solution of monolayers of organic substances. This method has been used by them for studying the properties of adsorbed layers of protein (1), enzymes (3), and immunological substances (1).

By adsorbing on barium stearate films of sufficient thickness to give interference colors, a rough estimate of the thickness of the adsorbed layer can be obtained by color comparison with films of known numbers of layers. A more precise method has been described by Blodgett and Langmuir (4) and Blodgett (5) in which the increase in thickness is calculated from the change of angle of incidence of the ordinary Rs[1] ray of sodium light for minimum reflectance.

Using the less accurate method of color comparison, Shaffer and Dingle (6) have investigated the antigen-antibody reactions between adsorbed monolayers of egg albumin and anti-egg albumin and between pneumococcus polysaccharide and antipolysaccharide specific antibody. They used whole immune serum for their studies.

Because of Shaffer and Dingle's unexpected results, we have undertaken a reinvestigation of the pneumococcus antigen-antibody reactions on adsorbing surfaces using the more precise method (4, 5) and antibody solutions purified by dissociation according to the method of Heidelberger and Kendall (7). In addition we have made measurements on the diphtheria toxin-antitoxin system. The results we have obtained are not in agreement with those of Shaffer and Dingle or those

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[1] The plane of polarization of the Rs ray is perpendicular to the plane of incidence.

of Langmuir and Schaefer (1) for toxin and antitoxin. The nature of these discrepancies and their possible explanation will be discussed later in this paper.

Materials and Methods

The three types of pneumococcus polysaccharides used, hereafter referred to as S I, S II, and S III, were prepared by the method of Heidelberger, Kendall, and Scherp (8).[2]

TABLE I

Analysis of Dissociated Antibody Preparations

Serum No.	Symbol	Species	Type	Nitrogen per cc.	Antibody nitrogen per cc.	Antibody nitrogen
				mg.	*mg.*	*per cent*
715-13	AH I$_1$	Horse	I	0.298	0.220	74
733-2*	AH I$_2$	"	I	0.151	0.066	44
701-20	AH II$_1$	"	II	0.183	0.083	45
701-19*	AH II$_2$	"	II	0.136	0.116	·85
536-11	AH III	"	III	0.351	0.202	58
J-5	AR I	Rabbit	I	0.271	0.102	38
J-6	AR II	"	II	0.142	0.091	64
J-7	AR III	"	III	0.116	0.090	78

Antibodies prepared from serums marked with an asterisk were obtained by dissociation of specific precipitates formed from solutions of Felton precipitates. Other serums were used without preliminary purification.

The solutions of Type I, II, and III horse and rabbit antipneumococcus antibodies were prepared by dissociation of washed specific precipitates, formed in the region of slight antibody excess, with 15 per cent sodium chloride at 40°C. according to the method of Heidelberger and Kendall (7). The per cent of specifically precipitable nitrogen was determined for each sample of dissociated antibody studied. These analyses are summarized in Table I. All protein solutions were finally dialyzed against M/15 phosphate buffer, pH = 6.9, containing 1:10,000 merthiolate. Solutions of S I, S II, and S III containing 0.25 per cent, 0.06 per cent, and 0.05 per cent carbohydrate respectively were made up in the same buffer.

The highly purified diphtheria toxin used was prepared by one of us (9) and has recently been found to show homogeneous sedimentation in the ultracentrifuge

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[2] We are greatly indebted to Dr. Heidelberger for presenting us with samples of highly purified S I (123-4), S II (83E), S III (105-7).

as well as homogeneous migration by electrophoresis (10).   An electrodialyzed, antitoxic pseudoglobulin preparation was used which was practically homogeneous by ultracentrifugation and 35 per cent precipitable by diphtheria toxin.

Films for conditioning and adsorbing were deposited on polished stainless steel slides by dipping through a stearate monolayer spread on a substrate 3×10^{-5} molar in $BaCl_2$, 10^{-3} molar in Michaelis' Na acetate-Na veronal buffer, pH 7.4 (11).   Under these conditions Y layers are deposited.   Castor oil was used to keep the monolayer under compression.

The stearate was deposited, by varying the depth of dip of the slide, to give films graduated in steps of two from 41 layers at the upper to 51 layers at the lower end.   The purpose in building a stepped film for adsorption is to provide a series of bands of varying reflecting power for polarized monochromatic light. By properly choosing the angle at which the film is viewed a pair of adjacent steps can be found of equal brightness.   For an unconditioned film the 47 and 49 layer bands match in brightness for the Rs ray under sodium light at an angle of incidence of about 71°.   On increasing the total thickness this angle increases.   If the thickness increase is large a match may more easily be obtained between a pair of steps of fewer stearate layers.   In calculating the total thickness increase this shift of the matched bands as well as the angle of incidence must be taken into account.   Thicknesses were calculated from the following equation:

$$T = \frac{\lambda}{4\sqrt{n^2 - \sin^2 i}}$$

in which T is total thickness, i the angle of incidence, $\lambda = 5893$ Å the average wave length of sodium light, and $n = 1.495$ the refractive index of barium stearate given by Blodgett (5).

Conditioning of the surface for adsorption was accomplished by the improved Langmuir-Schaefer method (2) which consists of successive immersions for 30 seconds in 10^{-3} molar thorium nitrate and dilute potassium silicate solutions followed by rinsing in distilled water and drying.

The adsorbate was applied according to the following technique: The slide was flooded with M/15 Sørensen phosphate buffer solution pH 6.9; a few drops of the adsorbate solution were added; then the mixture stirred for 30 seconds by moving a glass rod, in contact only with the upper surface of the solution, back and forth across the slide.   The preparation was rinsed first with M/15 phosphate buffer, then with distilled water and dried.   Application in this manner was repeated until the thickness became constant within the accuracy of the method, that is to ±3 Å, the equivalent of 0.5 degrees change in the angle of incidence at minimum reflectance of the monochromatic light.   The next adsorbate solution was then applied by the same procedure.   We have found it important to keep the composition of the solution in contact with adsorbing surface constant during the adsorption process in order to obtain consistency and reproducibility of thickness measurements.   A possible explanation of this necessity is to be found in a recent paper by Langmuir and Schaefer (12).

The Pneumococcus Capsular Carbohydrate-Antibody System

Pneumococcus antibodies occur in the euglobulin fraction of horse serum. When adsorbed on properly prepared slides an average

TABLE II

Reactions between Pneumococcus Specific Carbohydrates and Horse Antibodies

For order of application, read down

	Adsorbate	Thickness increase Å ± 3								Adsorbate	Thickness increase Å ± 3		
Series I	S III						-2		Series II	AH I₂	47	45	
	S I			-2						S III	0		
	AH I₁	42	50	47	56	60	48	40		AH III	-1		
	S I	-1	1	0				0		AH I₂	1		
	S II				-3					S I	1	-1	
	S III					-2	-1			AH I₂	17	20	
	AH I₁	36	45	62	6	8	11			S I		0	
	AH III							3		AH II₂		-1	
	S I	-7		-28						AH I₂		18	
	AH I₁	64		105				48					
Series III	S II	-1							Series IV	AH II₂	43	40	37
	AH II₁	46	36	40	31	29				S II	-2	-1	-2
	S I				2					AH II₂	23	25	30
	S II	3	1	0						S II	-1	-2	-2
	S III					3				AH I₃	1		
	AH II₁	11	4	7	4	5				AH II₂	46	57	46
	AH I₁			4									
Series V	S III	2											
	AH III	53	49	48	50								
	S I			-2		-1							
	S II				1		1						
	S III	-1	-1										
	AH III	45	48	6	11	51	46						
	S III		-3										
	AH III		62										

Each column in each series represents a complete experiment. Blank spaces indicate no treatment with the corresponding adsorbate.

thickness of 45 Å was obtained with an average divergence regardless of sign from this value of 6 Å. No systematic difference between the three types of horse antibodies was observed. None of the three

carbohydrates gave measurable adsorbed films on the conditioned stearate. When applied to an underlying layer of type specific antibody, however, they produced changes in the surface, without significantly increasing the total thickness, which caused the adsorption of a second layer of the homologous antibody of approximately the same thickness as the first layer provided the antibody solution contained a sufficiently large percentage of specifically precipitable nitrogen.

TABLE III

Reactions between Pneumococcus Specific Carbohydrates and Rabbit Antibodies

For order of application, read down

	Adsorbate	Thickness increase $\text{Å} \pm 3$					Adsorbate	Thickness increase $\text{Å} \pm 3$				
Series VI	AR I	15	16	33	32	Series VII	AR II	15	32	20	15	12
	S I	−2	.1	1			S II	0	2	1	1	1
	S II				2		AR II	6	7	6	10	10
	AR II	1	0				S II				2	0
	AR I		12	29	3		AR II				28	36
	S I		−2		0							
	AR I		17		21							
Series VIII	AR III	15	16	20	19							
	S III	0	0	0	0							
	AR II			1	2							
	AR III	27	29	19	20							
	S II				2							
	AR II				2							
	S III	−2	0	−2								
	AR III	45	48	28								

Each column in each series represents a complete experiment. Blank spaces indicate no treatment with the corresponding adsorbate.

This "sandwiching" effect (Table II) can be carried to a third layer of antibody, but generally at this stage the deposition becomes irregular making thickness measurements difficult. It is evident that the sandwich effect is specific from control experiments in which all attempts to obtain cross reactions failed as seen in Table II.

Evidence that the specific carbohydrates are not adsorbed on conditioned stearate surfaces from buffer solutions at pH 6.9 to produce even very thin layers is provided by the failure of any type

due to the fact that they applied whole immune serums to dry slides and to the possibility of adsorption from whole serums of large aggregates the existence of which have been reported by Goodner, Horsfall, and Bauer (15).

Molecular weights of proteins may be calculated, by the use of dissymmetry factors, from the thicknesses of adsorbed layers assuming the layers to be only one molecule thick.   However, because of certain features of this calculation the results should be interpreted cautiously. Thus, calculated molecular weights are extremely sensitive to variation in measurements of the thickness of the adsorbed layer because they are proportional to the third power of molecular diameters.   Also, axial ratios are very sensitive to variation in dissymmetry factors.

TABLE IV

Non-Specific Reactions

For order of application, read down

Series IX		Series X		Series XI	
Adsorbate	Thickness increase Å ± 3	Adsorbate	Thickness increase Å ± 3	Adsorbate	Thickness increase Å ± 3
AR I	15	AR II	15	AH I₁	42
AH III	24	AH II₁	27	AH III	3

Each column represents a complete experiment.

Nevertheless we present the following comparisons, assuming that the adsorbed molecules are cylindrical and lie with their long axes parallel to the adsorbing surface.   Kabat (16) has shown that pneumococcus antibodies are highly asymmetrical molecules having dissymmetry constants of 2.0 for horse antibody and about 1.5 for rabbit antibody. These values give from Perrin's equation (17) axial ratios of approximately 1/20 and 1/10 respectively.   Molecular weights calculated from these axial ratios and the average thicknesses of adsorbed layers are for horse antibody, 45 Å thick, approximately 1,300,000 and for rabbit antibody, 20 Å thick, approximately 57,000.   The molecular weights given by Kabat from sedimentation and diffusion constants are 990,000 for horse antibody and 157,000 for rabbit antibody. Making the same calculations with Shaffer and Dingle's values, we

get for horse antibody giving adsorbed layers by their technique 240 Å thick a molecular weight of 190,000,000, and for rabbit antibody with layers 100 Å thick 6,700,000.   Even if we assume that the molecules in Shaffer and Dingle's experiments are oriented with their long axes perpendicular to the adsorbing surface, an unlikely situation, the calculated molecular weights are not in any better agreement with those of Kabat.   Thus for horse antibody we get 23,000 and for rabbit antibody 6,800, values which are much too small.   Adsorption of aggregates remains, therefore, as the only explanation of their results.

Assuming the dissymmetry factor for antitoxic pseudoglobulin to be the same as normal horse pseudoglobulin, its molecular weight may be calculated by the same method.   Using the factor 1.4 given in a paper by Svedberg (18) and the average thickness of adsorbed layers of 23 Å, a molecular weight of 73,000 is obtained.   The value given in Svedberg's paper for normal horse serum globulin calculated from the sedimentation constant is 150,000.

From Lundgren, Pappenheimer, and Williams' data (10) the dissymmetry factor for diphtheria toxin was calculated as 1.25, and from Perrin's equation this value gave approximately 1/5 for the axial ratio.   Using this ratio and the average thickness of adsorbed layers of 33 Å, the molecular weight of toxin was determined as about 120,000, which is in fair agreement with 72,000 given by these authors from sedimentation and diffusion constant measurements.   Our thicknesses are, therefore, in no way inconsistent with the present knowledge regarding molecular weights.

Our experiments with the pneumococcus system would appear to give support to the lattice theory of antigen-antibody precipitin reactions of Marrack (19) and Heidelberger (20).   In the protein systems studied by the quantitative precipitin method and in the ultracentrifuge, no evidence has been obtained that antigen and antibody can unite in a proportion of more than one molecule of antigen per molecule of antibody, although it is clear that many molecules of antibody can react with one of antigen (Heidelberger, 21).   The sandwich effect obtained with the pneumococcus system suggests that antibody as well as antigen can be "multivalent" in conformity with the views of Marrack and Heidelberger.

Langmuir and Schaefer with one of us (1) have reported obtaining

with diphtheria toxin and antitoxin alternate adsorbed layers 36 Å and 75 Å thick respectively. They state that the alternation can be continued indefinitely. Our value for adsorbed toxin is in good agreement with this, while our value for purified antitoxin on toxin is somewhat less. As for indefinite alternation of layers we have been completely unable to corroborate this finding. The most we could obtain was a layer of antitoxin on toxin but not the converse. A non-specific deposition might be the explanation of this discrepancy for Langmuir and Schaefer used a pseudoglobulin preparation only 9.6 per cent precipitable by toxin, for their experiments. We have already pointed out the necessity of using purified preparations and have shown that normal pseudoglobulin will deposit on toxin.

SUMMARY

By adsorbing antigens and antibodies on barium stearate multilayers immunological reactions at surfaces have been studied.

Pneumococcus polysaccharide specific antibody systems using purified antibodies from both horse and rabbit sera were investigated. The polysaccharides failed to show visible adsorption, but by alternate treatment with antibody and polysaccharide several layers of antibody could be specifically deposited.

With the diphtheria toxin-antitoxin system antitoxin was found to adsorb to layers of toxin but not conversely. The reaction, however, was not specific.

Molecular weights calculated from the thickness of adsorbed protein layers, using dissymmetry factors, roughly correspond to molecular weights calculated from sedimentation and diffusion constants.

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STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

VI. EXPERIMENTS ON THE SENSITIZATION OF GUINEA PIGS TO POISON IVY

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PLATE 42

One of the open questions concerning skin sensitization of the "contact dermatitis" type to simple chemical compounds is in which way sensitivity spreads all over the integument when only a given area is treated. This subject has been considered in a paper by Simon (1) and studied experimentally. The skin of guinea pigs was treated with concentrated nitric acid in such manner that a complete belt severed the continuity of the epidermis. Poison ivy extract was applied on the posterior part of the animals and after 10 days the anterior and the posterior parts were tested; both halves were seen to be sensitive. According to the author the evidence "indicates that the route of distribution is not confined to the epidermis," and suggests that the spread is due to distribution of altered allergen by the blood stream or the lymphatic system.

Furthermore, Simon observed that no significant difference exists in the degree of sensitivity between the area used for the sensitizing application and other parts of the skin, and by timed excisions found that such removal of the treated area did not interfere with the general skin sensitization provided it was performed later than 18 to 24 hours after the application of ivy extracts. He also found that sensitivity was first seen after a latency period of from 4 to 6 days.

In contrast to the result of Simon, Straus and Coca (2) reported that in monkeys severance of the continuity of the skin some distance above the elbow and application of poison ivy extract to the forearm

resulted in sensitization of this part only and prevented general skin sensitization. The experiment was also performed in reversed fashion. In the authors' opinion, their results suggest that the spread of sensitization is attributable "probably to a diffusion of the oily excitant through the oily substances normally present in the skin," in harmony with a hypothesis previously advanced by Coca (3, 2).

Similar results have recently been described in a preliminary communication by Schreus (4) who treated guinea pigs with dinitrochlorobenzene, a substance found in this laboratory (5, 6) to sensitize these animals. This author assumes spread by way of the intercellular bridges connecting the cells of the epidermis.

Further investigation of the subject was desirable, particularly in view of the lack of agreement in the reported experiments.

General Methods

Male albino guinea pigs from healthy stock, caged separately, were employed in the experiments; since operative procedures were to be carried out, rather heavy animals were commonly selected (500 to 600 gm. weight). For uniformity as regards the period allowed for sensitization (10 to 14 days) a group included only the animals operated upon within a 3 day period, and these were tested at the same time; in instances of comparisons between differing methods, the operations in question were done as far as practicable in alternation to avoid technical bias. All operations were conducted under deep ether anesthesia.

Sensitizing Application.—Poison ivy extract Lederle,[1] supplied as a 12½ or 13 per cent solution in acetone of extractives from *Rhus toxicodendron radicans*, was used to sensitize guinea pigs by application to the skin on a clipped (frequently also shaved) site, spread evenly over a circular area 10 to 11 mm. in diameter with the tip of a thin glass rod. The doses ranged from 0.05 cc. of the undiluted extract to 0.025 cc. of a 1:5 dilution in acetone or less, delivered as a series of micro drops (0.05 cc. = 10 to 13 drops) from a gauge 26 Luer needle with tip ground flat, attached to a 1 cc. Dewitt and Herz record (tuberculin) syringe where the amount discharged can be accurately controlled by a check-nut on the plunger shaft. Upon full evaporation of the solvent, an even, brownish, waxy film remained on the skin.

Rather elaborate precautions were taken in the case of experimental skin barriers to ensure that no ivy material could come into contact with other parts of the skin than the desired field. To this end, several layers of bandage gauze and an outer covering of crepe tissue paper were used to shield the body, the site

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[1] This material came to us through the courtesy of Dr. Arthur F. Coca of the Lederle Laboratories. It may be recalled that the active principle of poison ivy is urushiol, a catechol with a fifteen carbon, unsaturated side chain (*cf.* 6).

of application being exposed through a hole in the coverings, which were firmly held against the skin, and likewise all instruments used after application of the ivy extract were not handled again until they had been cleaned chemically (with alcohol, and then with potassium permanganate). Finally the deposit was covered with a 14 mm. square of cellophane (cut from cellophane tubing, each of the two adhering layers being about 0.002 inch thick) cemented to the skin, along its margin, by means of duo liquid adhesive (Johnson and Johnson); over this was affixed a disc, about 30 mm. in diameter, of finely woven linen, the adhesive first being applied both to the periphery of the linen and to the skin outside the cellophane patch.

On the 4th or 5th day the ivy was removed, either, in early experiments, by cleaning the site with olive oil and acetone and painting with aqueous permanganate solution, or by excision, *in toto*, of the treated skin and its protective coverings, as described below.

Test for General Cutaneous Sensitivity.—On the 10th to 14th day following the original application of ivy extract to the skin, various areas were clipped with an electric clipper, and on these sites single drops (approximately 1/60 cc.) of freshly prepared dilutions in 95 per cent alcohol of the extract were allowed to fall from a fine capillary pipette. The drops were at once spread with the tip of a thin glass stirring rod over an area about 10 to 11 mm. in diameter and dried in a gentle current of air from a small electric blower; the sites were marked by touching the centers with the moistened tip of an indelible pencil.

The highest concentration of ivy causing no, or occasionally a slight reaction with normal animals was determined for each lot of extract (a 1:15 or 1:20 dilution), and was applied to the flank and to 2 sites on the back while areas close to these were tested with half the concentration (see Figs. 1, 2); and one-half and one-third concentration were used on the belly.

First readings were taken 24 hours later, some time after the sites had been cleaned with pledgets of cotton wet with acetone; second readings were made the day following. With the most sensitive animals, the reactions are strongest at 24 hours, but then turn brownish and begin to fade, while animals of lesser sensitivity may exhibit higher reactions at the second reading. In our experience, there was no difficulty in recognizing the difference between even moderately sensitized animals and normal controls similarly tested.

The intensity of the reactions was designated as follows: $+++++$, pink or dark pink, sometimes slightly elevated; $+++$, pink, but either somewhat pale or macular; $++$, between faint pink and pale pink in color; $+$, faint pink; $\pm$, faint pink ring; tr., trace; f.tr., faint trace.

EXPERIMENTAL

In order first to determine, under the conditions of our experiments, how long the active material must remain in contact with the skin to induce hypersensitiveness, and the length of the latency period before sensitivity appears, experiments similar to those of Simon (1; *cf.* 7)

were made.   As regards the first question, sensitization was found to result if the treated area was extirpated later than 8 to 12 hours following the application (Table I).

TABLE I

Composite Table

Sensitization with poison ivy extract in relation to the excision of treated skin areas at different times (see text).   The readings recorded were made 24 hours after application of the test doses on the 11th day; also observations at 48 hours are given (within parentheses) when there was an increase over the earlier reactions.

No.	Time before excision	Reactions to ivy extract			
		Dorsum		Flank	
		Dilutions		Dilutions	
		1:15	1:30	1:15	1:30
	hrs.				
1	4	f.tr.	0	0	0
2	4	f.tr.	0	f.tr.	0
3	6	0	0	0	0
4	6	0	0	0	0
5	8	± (+++)	± (±)	± (++)	tr. (++)
6	8	0	0	0	0
7	12	++++	++	++++	+
8	12	++	+	+++	+++
9	16	++ (+++)	± (++)	± (+++)	± (+)
10	16	++++	++++	++++	++++
11	16	++++	+++	++++	+++
12	16	+ (+++)	tr. (±)	+ (++++)	± (+)
13	24	++++	+++	+++	+++
14	24	++++	+++	++++	+++
		Normal controls			
15		0	0	tr.	0
16		f.tr.	f.tr.	0	0
17		f.tr.	f.tr.	tr.	0
18		± (tr.)	0	0	0
19		tr.	tr.	±	tr.
20		tr.	0	tr.	0

A linen ring having an outer diameter of about 26 mm. and an 18 mm. opening was affixed to the skin in the sacral region of the back by means of the liquid adhesive mentioned.   The body of the guinea pig was shielded and only the skin

within the ring was exposed; on this site 0.03 cc. poison ivy extract was put on a circular area approximately 12 mm. in diameter. Upon evaporation of the acetone the residue was covered by a 14 mm. square of cellophane cemented at the corners to the skin and above it a linen disc 26 mm. in diameter was attached similarly to the linen ring. Cardboard collars (adapted from (8)) were fixed around the neck to keep the coverings undisturbed. After varying intervals the skin bearing the ivy and coverings was excised 1 to 2 mm. beyond the linen ring (with anesthesia); in this way not only the ivy-treated area but also a wide margin (8 to 11 mm.) outside was removed and the defect was dressed with thymol iodide. The animals were tested for sensitivity on the 11th or 12th day.

The somewhat shorter period of contact as compared with that of Simon possibly is due to the use of a larger dose of ivy extract or to some other technical difference. The necessary time of contact may be that required for absorption of a sufficient quantity of the incitant (which would vary according to such conditions as the amount and size of the treated area) or possibly an interval during which some chemical process takes place in the skin.

In our experiments on the latency period, hypersensitivity became manifest to tests made 5 days after the ivy was placed on the skin. Remarkably, the onset was quite regularly sudden with well developed reactions to the larger test dose present at the 24 hour reading and in tests made after 6 days the reactivity already was maximal (Table II).

The sudden appearance of hypersensitivity was further emphasized by the coincident and equal reactivities seen on the 6th day to the applications of the 5th day (one day reading) and the 4th day (48 hour reading), the latter having produced no significant reaction at 24 hours.

As pointed out before, the main issue of our investigation was rather the question whether an epidermal pathway is a necessary condition for the spread of sensitivity from one site to the whole body. In the first experiments, an area of skin on the back was isolated during deep anesthesia by means of a circular cautery burn roughly 5 mm. wide and deep enough to destroy the epidermis with certainty (cf. 1), and then was treated with ivy. The site was covered to avoid mechanical transfer and after 4 to 5 days was carefully freed from the incitant, or the whole isolated area was excised. When the animals were tested 6 to 8 days later, all were definitely hypersensitive, in varying degrees.

While this experiment in agreement with Simon's appeared to show

that interruption of epidermal continuity did not prevent general sensitization, we desired to attempt the experiment under more rigorous conditions.   For this purpose a deep circular cut down to the muscles of the trunk was made on the back (or flank), thus isolating an island of skin, the edges, through contraction, being separated by a wide gap.   By excision of additional strips of skin, depending upon the location, the gap was made uniformly wide (7 mm. or more).

TABLE II

Latency period preceding hypersensitivity to poison ivy.   0.05 cc. extract was applied to the sacral region and left for 4 days, covered with cellophane and linen patches; the residue then was removed with solvents and the site swabbed with $KMnO_4$ solution.   Test applications on the back were made at the times noted after the ivy was placed on the skin, and concomitantly on additional animals as controls; the test doses were removed on the succeeding day.   The table shows the 24 hour readings of the reactions to both dilutions, 1:20 and 1:40, separated by the slant line.

No.	Reactions to ivy extract applied at stated intervals following the primary administration					
	48 hrs.	3 days	4 days	5 days	6 days	7 days
21	0 / 0	f.tr. * / 0	tr. / 0	± / 0	++++ / ++	++++ / +++
22		0 / 0	0 / 0	++++ / +++	++++ / ++	+++ / +
23		0 / 0	0 / 0	++++ / +++	++++ / +++	
24		0 / 0	0 / 0	+++ / +	++++ / +±	
25		tr. * / 0	tr. / 0	+++ / +	++++ / +++	++++ / +++
26			f.tr. * / 0	+++ / tr.	++++ / +++	++++ / +++
27			f.tr. * / 0	++++ / +	++++ / +++	++± / +
28				+ / 0	++± / ±	+++ / +
29				++ / 0	+++ / +	+++ / ++
30				± / tr.	++++ / +++	++++ / ++±
31					+++ / ±	+++ / +
32					++++ / +	+++ / ++
33						+++ / +
34						++++ / ++++

* These reactions were less, or not greater than those exhibited by one or more of the 3 or 4 controls tested at the same time; on the average, about half of the controls showed trivial reactions (f.tr. or tr.) to the higher concentration.

With this technique the results were now different, sensitization being obtained in some cases but inconsistently and, with few exceptions, of low degree.   In order to obtain more decisive results, several different procedures were tried.   Finally definitely positive or negative sensitization effects across a dermal barrier were obtained almost regularly, depending upon whether or not the thin muscle layer underlying the cutis, the panniculus carnosus, was severed as well as the skin.   A cut was made upon the flank circumscribing an area of skin; poison ivy

extract was applied in the center of this area, which then was carefully covered to prevent transfer of the active material to other parts of the integument. 4 or 5 days later the skin island, together with the protective coverings, was removed. By inspection it was easy to make sure at this time that no appreciable epithelial growth had taken place and that the edges of the epidermis were still wide apart; this macroscopic observation was confirmed by histological examination. Between the 10th and 14th days the animals were tested on various sites (see Figs. 1 to 4). The operations described were well tolerated, and within the 14 day period there was an actual gain in weight, averaging from 15 to 40 gm. with the different groups of animals.

The procedures will be described in some detail because they are of importance for obtaining clear cut results.

Flank Islands with Panniculus Carnosus Intact.—The position of the desired island was sketched with pencil on the clipped and shaved left flank, with the guinea pig in resting posture; after the animal under ether anesthesia had been fixed to the board on its side so as to maintain a right angle between trunk and extended hind limbs, without displacement of the loose flank skin, the outline was completed with the help of an ovoid stencil, 43 mm. by 37 mm. along the axes. (It might prove possible to make use of smaller islands in this type of experiment.) In an animal of about 550 gm. weight, the anterior midpoint of the line was, for instance, about 15 mm. posterior to the scapular angle. A second line was then drawn, to give a nearly rectangular figure, situated outside the first by 5 mm. at the dorsal and ventral midpoints, 6 to 7 mm. at the anterior and posterior midpoints, and separated by as much as 10 mm. along the diagonal diameters midway between dorso-ventral and antero-posterior diameters. (This technique assists in obtaining an island of circular shape, and a uniform width in the denuded ring.)

After the flank was sponged with alcohol, shallow incisions were made along both lines and carried cautiously down towards the panniculus carnosus, which appears as a grayish layer below the firm white connective tissue; the intervening skin was then carefully and sharply dissected from the panniculus, the procedure being facilitated by use of a binocular loupe. The field of dissection was kept moist with wet dressings, to reveal any residual bridges of dense connective tissue. Relatively little bleeding occurred. Upon contraction, the skin thus isolated becomes a nearly circular island of about 35 mm. diameter, surrounded by a ring-shaped defect about 8 mm. wide (Figs. 4, 5). When correctly done, which requires attention to detail, the operation will leave patent for the greater part the lymph vessels extending across the panniculus, as may be evidenced by intracutaneous injection into such islands of solutions of dyes (9) e.g., pontamine sky blue 6 B.

With the body protected against accidental contact, the ivy extract was applied to the center of the island in the manner described previously; then the cellophane and linen coverings were cemented to the island over the ivy material.

The wound was kept dry and clean by applying thymol iodide (Merck); this treatment was repeated several times, and thereafter once daily.

The claws of the hind feet were covered with boots of adhesive tape and, besides, the animals were prevented from disturbing the bandage by attachment of a cardboard collar or, preferably, by the following device: by means of a strip of cotton twill tape attached to an adhesive tape band around the right thigh and then affixed to a collar of iron wire covered with soft rubber tubing, worn around the neck, the leg was advanced toward the neck sufficiently to keep the trunk bent slightly sidewise, away from the flank island. This arrangement, maintained until subsequent excision of the islands, prevented narrowing of the defect and kept the cut surfaces from adhering, without otherwise interfering with the free movements of the animal. The isolated skin was surrounded by an even, circular moat, which remained dry (but for exceptional bleeding) and in which a crust appeared about the 3rd day. Frequent inspections were made, and occasionally blood clots or dried exudate were removed. (In the early experiments where cardboard collars were employed, free movements of the flank sometimes led to a sticking together of the opposing cut surfaces: whenever this occurred, the animals were withdrawn from the experiment.)

Flank Islands with the Panniculus Severed.—The location of the islands and the stencil used were the same as described above. The incision was extended downwards through the panniculus, whereupon the muscle contracted to give a gaping moat. Consequently, only narrow (3 to 4 mm.) strips of skin opposite the anterior and posterior margins of the island had to be removed in order to secure the same outer diameters of the ring (about 50 by 50 mm.) which obtained in the operations where the panniculus was left intact. Blood vessels were tied off when necessary. The isolated skin areas thus created measured about 29 mm. along the antero-posterior axis and 32 mm. dorso-ventrally (Fig. 3). (When dye is injected intracutaneously into such islands, it infiltrates the tissue and in some measure oozes out into the moat.) Application of ivy extract, protective coverings, and postoperative care followed the procedures in the section above; to be emphasized is the collar-to-leg method of restraint in order to maintain an even and dry moat despite the loose connective tissue between panniculus and trunk muscles. (By frequent application of thymol iodide, the wound dries satisfactorily, although a small area along the ventral surface may remain moist for a day or two.)

Excision of the Islands.—On the 4th day, exceptionally on the 5th, the hair near the island was clipped and, with the animal under deep ether anesthesia, the isolated skin with ivy material and coverings undisturbed was removed by dissection, usually sparing the panniculus carnosus. Vessels were clamped off and tied. Finally, the whole area was packed with thymol iodide and covered with sterile cotton cloth, which was cemented to the skin. In the case of the

islands where the skin muscle had been cut, there was edema by the 2nd or 3rd day, and during excision free fluid was present; the panniculus itself and the underlying tissues appeared thickened.

From the results of the two sorts of operations (Table III *a*), it is seen that of 25 guinea pigs in which an area of the skin had been isolated with severance of the skin muscle and the superficial lymph vessels, only two attained a marked degree of sensitivity and a few others gave some evidence of a slight sensitization; where the panniculus and the lymphatic trunks passing over its surface were spared, however, and this despite the need of care in the operation, all of the 25 animals became sensitive, almost regularly in high degree and comparable to the sensitization elicited by like treatment of normal animals (treated with group D, Table III *a*); one showed a low grade sensitivity and some a moderate hypersensitiveness. It will have been noted that a large dose was selected for the sensitizing application in these experiments (0.03 or 0.05 cc. of undiluted ivy extract); by this means the demonstration of the difference between the two operations was made more striking, but a large amount was not necessary, for smaller doses of ivy extract applied to the skin islands with intact skin muscle were sufficient to sensitize (Table III *b*).

It will be clear that the positive results can meet with no objections on technical grounds, particularly the possibility of contamination of the skin outside the island, a matter necessarily to be considered with so highly active a substance. Apart from the elaborate precautions taken, the two types of experiments were made in alternation and with identical technique (except for the difference in the cutting), and furnished convincing controls for one another.

It should be noted that a not inconsiderable percentage of positive sensitizations was obtained, in most cases, as mentioned, not of high degree, when "deep" cuts were made in other locations (principally on the back) than the one described; from some experiments with dye injections we are inclined to believe that this may be due, at least in part, to incomplete interruption of lymph passage.

Straus and Coca (2) as well as Schreus (4) have reported that in their experiments treatment of isolated parts of the skin resulted in local sensitization. Indeed that such a condition can occur seems evidenced by certain clinical experiences. Our somewhat limited attempts to achieve a local sensitivity restricted to a segregated area have so far not yielded a definitely positive result: we obtained either no sensitization at all, or a hypersensitiveness which included the

Sensitization in relation to a skin defect encircling the site of application of poison ivy on the [...] severed as well. The islands of treated skin were excised on the 4th (rarely the 5th) day, and [...] A, 0.03 cc. ivy extract No. 1 was applied to the isolated skin, and the animals were finally tested [...] No. 2 was used for sensitization, and dilutions of the same extract were employed for the tests. [...] on two regions of the skin are shown in the table, the readings recorded being those made at 24 and [...]

		Ivy applied to skin islands, panniculus carnosus intact						Ivy applied to ski[...]
No.	Interval before test	Reaction on dorsum		Reaction on flank		No.	Interval before test	Reaction
		1:20	1:40	1:20	1:40			1:20
	days						*days*	
								Gr[...]
35	13	+, +++	±, ±	±, +++	0, tr.	41	13	0, 0
36	13	++, ++++	±, ±	±, ±	tr., tr.	42	13	0, 0
37	12	++, +++	±, ++	+, ++	tr., +	43	12	0, 0
38	11	++++, +++	+++, ++	+++, +++	±, ++	44	11	0, 0
39	11	+++, +++	±, ±	+, +++	±, ±	45	11	f.tr., 0
40	10	+++, +++	+++, +++	+++, ++	++, ++	46	10	0, 0
		1:15	1:30	1:15	1:30			1:15
								Gr[...]
53	14	++++, ++++	+++, +++	+++, +++	+, +++	61	13	±, ±
54	12	++++, ++++	++++, ++++	+++, +++	+++, +++	62	13	±, +
55	12	++++, ++++	+++, ++++	++++, +++	+++, +	63	12	tr., tr.
56	12	++++, ++++	++++, ++	++++, ++++	+++, +++	64	12	+, +++
57	11	++++, ++++	++, ±	++, ++	±, ±	65	11	f.tr., f.tr.
58	11	++++, ++++	++++, ++++	+++, +++	+++, +++	66	11	0, f.tr.
59	11	+, ++	tr., ±	±, ±	0, 0	67	10	±, ±
60	11	+++, ++++	+++, ++++	++++, ++++	++, ++	68	10	0, tr.
								Gr[...]
73	14	+++, ++++	++, +++±	++++, ++++	++, ±	78	14	±, tr.
74	14	++++, +++	++++, +++	++++, ++++	++++, ++++	79	14	±, ±
75	13	++, +++	. 0, tr.	±, ++	0, 0	80	13	tr., f.tr.
76	12	++++, +++	++++, +++	++++, +++	++++, +++	81	13	+, ++
77	12	++++, ++++	++++, ++++	++++, ++++	++++, +++	82	13	f.tr., f.tr.
								Gro[...]
86	12	+++, +++	+, +	+++, +++	++, ++++	92	12	tr., 0
87	12	++++, ++++	+++, +++	+++, ++++	+, ++	93	12	f.tr., 0
88	12	++++, ++++	+++, +++	++++, ++++	++++, ++++	94	12	+++, +++
89	12	++++, +++	+++, ++	++++, ++++	++++, ++++	95	11	f.tr., 0
90	11	++++, ++++	++++, +++	++++, ++++	++++, ++++	96	11	tr., 0
91	10	++++, ++++	++++, +++	+++, +++	+++, +++	97	10	tr., tr.

II a

nk, (a) when the skin muscle is not involved in the operation, and (b) when the skin muscle is
en the 10th and 14th days the guinea pigs were tested for general hypersensitivity.   With group
th various dilutions thereof; on the remaining animals (groups B, C, D), 0.05 cc. ivy extract
ntreated animals were included as controls with each lot of guinea pigs tested.   The reactions
hours respectively.

ls, panniculus carnosus severed				Non-sensitized controls			
sum	Reaction on flank		No.	Reaction on dorsum		Reaction on flank	
1:40	1:20	1:40		1:20	1:40	1:20	1:40
A							
tr., 0	0, 0	0, 0	47	f.tr., ±	0, 0	f.tr., tr.	0, 0
0, 0	0, f.tr.	0, 0	48	0, 0	0, 0	f.tr., ±	0, 0
0, 0	0, 0	0, 0	49	0, 0	0, 0	0, 0	0, 0
0, 0	0, 0	0, 0	50	0, 0	0, 0	tr., 0	0, 0
0, 0	0, 0	0, 0	51	0, 0	0, 0	0, tr.	0, 0
0, 0	0, 0	0, 0	52	0, 0	0, 0	0, 0	0, 0
1:30	1:15	1:30		1:15	1:30	1:15	1:30
B							
tr., tr.	f.tr., f.tr.	0, 0	69	f.tr., f.tr.	0, 0	f.tr., ±	0, 0
tr., tr.	0, 0	0, 0	70	0, 0	0, 0	0, f.tr.	0, 0
0, 0	0, 0	0, 0	71	±, tr.	0, 0	f.tr., f.tr.	f.tr., 0
tr., tr.	+++, +++	f.tr., 0	72	f.tr., f.tr.	0, 0	±, tr.	0, 0
0, 0	0, 0	0, 0					
tr., f.tr.	±, ±	0, 0					
0, 0	f.tr., f.tr.	0, 0					
0, 0	0, 0	0, 0					
C							
tr., 0	0, 0	0, 0	83	0, 0	0, 0	0, 0	0, 0
tr., 0	±, tr.	0, 0	84	tr., ±	f.tr., 0		
0, 0	0, 0	0, 0	85	0, 0	0, 0	0, 0	0, 0
0, 0	tr., ±	0, 0					
0, 0	±, tr.	0, 0					
D							
0, 0	f.tr., 0	f.tr., 0	107	+, tr.	0, 0	tr., 0	0, 0
0, 0	0, 0	0, 0	108	0, 0	0, 0	0, f.tr.	0, 0
+, +	+++, +++	+++, +++	109	f.tr., f.tr.	0, 0	0, f.tr.	0, 0
0, 0	0, tr.	0, 0					
0, 0	0, 0	0, 0					
0, 0	tr., 0	0, 0					

Normal animals treated with ivy concurrently with group D and tested at the same time

No.	1:15	1:30	1:15	1:30
	Sensitization with 0.05 cc. of undiluted extract			
93	++++, ++++	+++, ++	+++, +++	+++, ++
99	++++, +++	++++, ++	++++, ++	++++, ±
100	++++, ++++	++++, +++	++++, ++	++++, +++
101	++++, ++++	++, ++	++++, ++++	++++, +++
102	++++, +++	++++, +++	++++, +++	++++, +
	Sensitization with 0.05 cc. of 1:100 diluted extract			
103	f.tr., 0	0, 0	++, +++	tr., 0
104	f.tr., tr.	0, 0	f.tr., 0	0, 0
105	+++, +++	+++, ++	++++, +++	++++, ++
106	±, 0	0, 0	f.tr., f.tr.	0, 0

isolated area to which the substance (ivy extract, 2:4 dinitrochlorobenzene) had been applied, and the whole integument as well. Furthermore, when poison ivy was applied to a site of the intact skin and tests were made after a suitable interval, the intensity of the reaction on the treated site was if at all only slightly stronger than elsewhere (cf. 1).

A counterpart to the experiments described is the sensitization of a segregated skin area (made by a "deep" cut on the flank) when the active agent is administered on another part of the skin. Since under these conditions the island can be sensitive also, the spread through the circulation either of the allergen, probably transported in a changed state, or of antibodies is indicated. The reactions on the island were relatively weak, which may be due to alteration of the tissues. These experiments bear some relation to the "belt operation" of Simon (1).

TABLE III b

Same as Table III a, except that a smaller amount of ivy extract was applied to the skin islands (0.025 cc. of a 1:5 dilution in acetone of ivy extract No. 2). The animals were tested on the 10th or 11th days with dilutions of the same extract.

	Ivy applied to skin islands, panniculus carnosus intact					Controls			
	Reactions on dorsum		Reactions on flank			Reactions on dorsum		Reactions on flank	
No.	Dilutions		Dilutions		No.	Dilutions		Dilutions	
	1:15	1:30	1:15	1:30		1:15	1:30	1:15	1:30
110	+++, ++++	++, +++	+, ++	±, +	116	tr., tr.	0, 0	tr., ±	0, tr.
111	+++, ++++	+++, ++++	+++, +++	+, +	117	f.tr., 0	0, 0	0, 0	0, 0
112	+++, +++	tr., tr.	+, +	±, ±	118	tr., tr.	0, 0	tr., ±	tr., f.tr.
113	+++, ++++	++, ++	++++, +++	±, ±	119	±, tr.	0, 0	0, 0	0, 0
114	±, ++	tr., 0	±, ±	tr., ±	120	tr., tr.	tr., f.tr.	±, +	tr., 0
115	++++, ++++	+++, ++	+++, +++	+++, ++					

We next proceeded to investigate whether a cut through the skin and the superficial muscle would also interfere with anaphylactic sensitization to a common protein antigen if injected intracutaneously into the isolated area. Actually this was the case when small doses (0.00005 cc., 0.0002 cc.) of horse serum were used (Table IV); with larger amounts (0.0006 cc. or more) such an effect did not occur. This may show that there are other ways by which high molecular substances can be distributed from a skin site than the lymphatic vessels that were severed along with the skin muscle in our experiments; indeed this idea may be considered for the infrequent instances of positive sensitization with ivy from flank islands similarly isolated.

The positive results just mentioned with large doses of horse serum

may possibly be ascribed also to leakage of some of the protein into the defect from the cut lymphatic trunks. For it was seen that when diluted horse serum (0.0025 cc. contained in 0.3 cc.) was applied with

TABLE IV

Hindrance by a defect in skin and panniculus of anaphylactic sensitization to horse serum injected intracutaneously in small amounts. Skin islands on the flank with severance of the panniculus carnosus, as described for poison ivy, were prepared in guinea pigs weighing between 450 and 500 gm., and 0.1 cc. of diluted horse serum was injected intracutaneously into the center of the isolated skin or in the corresponding position on normal animals as controls. The islands, together with underlying panniculus, were excised on the 4th day. After 21 or 25 days the animals were injected intravenously with 0.3 cc. of horse serum contained in a volume of 1.0 or 1.5 cc. Figures in parentheses indicate change in temperature (°C.).

Injection into skin islands, panniculus severed		Controls	
No.	Intravenous injection of horse serum	No.	Intravenous injection of horse serum
Sensitization with 0.0002 cc. serum			
121	No symptoms (+0.3)	127	Severe shock, recovered (−1.2)
122	Slight symptoms (−1.4)	128	† 9 min.*
123	" " (−1.0)	129	† 4 "
124	Severe shock, recovered (−2.8)	130	† 3 "
125	No symptoms (+0.5)		
126	" " (+0.9)		
Sensitization with 0.00005 cc. serum			
131	No symptoms (−0.4)	138	Moderate to severe (chronic type) (−3.5)
132	Very slight symptoms (+0.3)	139	† 4 min.
133	Slight to moderate symptoms (−0.5)	140	Slight to moderate symptoms (−2.3)
134	No symptoms (+0.8)	141	† 4 min.
135	" " (−0.5)	142	† 3 "
136	" " (+1.0)	143	Severe symptoms (chronic type) (−3.5)
137	Slight symptoms (−0.4)	144	† 3 min.

* The symbol † signifies death; the autopsy findings were typical in all cases.

a glass rod, slowly and with pauses, to the fresh wound surrounding flank islands that were made with severance of the panniculus carnosus, anaphylactic sensitivity frequently ensued, although not always with acute lethal shock (subsequent reinjection of horse serum as in

Table IV). It should be noted that the 0.0025 cc. amount used constitutes a rather large dose.

Similar exploratory experiments were then undertaken with another sensitizing substance of known structure, namely salvarsan (10), which induces skin sensitivity of a type different from the contact dermatitis of poison ivy. Here again, the barrier proved effective, for sensitization did not ensue when 0.15 mg. of salvarsan in 0.1 cc. was injected intracutaneously into flank islands (with the panniculus severed); the islands were excised on the 4th day, and tests for dermal sensitivity were made after 3 to 4 weeks by intracutaneous injection of a like amount.

DISCUSSION

From the experiments made in guinea pigs, which still leave problems for further investigation, the conclusion seems inescapable that continuity of the skin is not required for the development of general dermal sensitization to simple chemical compounds, since a broad defect in the entire thickness of the skin surrounding the area to which ivy extract is applied does not prevent hypersensitivity all over the skin. The significance of the question is clear from the literature reviewed. That there are differences as regards the route of distribution of the agent in various species would in our opinion seem quite improbable.

It is true that a "deep" cut down to the muscles of the trunk, made as described, inhibited sensitization almost regularly, and in this respect there is conformity with the results reported by Straus and Coca (2) for monkeys, and recently by Schreus (4) for guinea pigs. But upon a change in the experimental conditions the outcome was strikingly different, that is, when a strip of skin 5 to 10 mm. in width and comprising the whole thickness was excised in such manner as to spare the underlying skin muscle. The fact that the results differ depending upon the integrity of this muscle is apparently referable to the location of the lymph vessels draining the skin on the surface of the panniculus carnosus, with the consequence that cutting the muscle layer interrupts the lymphatics.[2] This can be demonstrated

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[2] The anatomy of these vessels and the lymph nodes in the guinea pig are well described by Keller (11) whose drawings picture the superficial ramification of the lymph vessels. In this connection we may quote the conclusion of McMaster (12) that "every intradermal injection is truly intralymphatic."

by injecting intracutaneously into a freshly isolated island a solution of a colloidal dye, such as pontamine sky blue 6 B, for when the muscle is severed the dye chiefly is held locally, penetrating into the connective tissue, and to some extent oozes out from the severed lymph vessels; whereas, if the muscle is left intact, one sees the dye streaming across the moat through the lymphatics, and shortly the superficial and even the deeper regional lymph nodes are found upon dissection to be blue.[3]

Concerning the reasons for the interference with sensitization through severance of the lymph vessels, the most obvious is prevention or hindrance of transportation of the active material, if the latter is not such as to pass easily into the blood stream. In this respect it may be pointed out that sensitizing substances of simple constitution are probably in many instances not carried as such but rather in the form of some sort of conjugates (cf. 6), and indeed some of the compounds by their very instability (diazomethane (13), acyl chlorides (6)) must react rapidly when brought into contact with tissues; with poison ivy, as Simon (1) has reported, and this holds in our experience for other sensitizers of simple chemical constitution, direct introduction of the extract into the blood stream fails to induce skin hypersensitivity, from which fact he suggests that the active agent, if it is distributed by the blood, must have undergone some prior transformation.

In support of mechanical causes in preventing sensitization from skin islands isolated by a "deep" cut are experiments which show that anaphylactic sensitization by proteins, when one injects not too large amounts into such islands, also is definitely impaired (Table IV). Aside from hindrance to lymph flow, loss of some of the protein by leakage into the wound will occur, as with dye injected intracutaneously into the island.[4] Whether much effective antigen is lost in this way is doubtful for dilute horse serum placed on the freshly made circular defect is in some measure taken up, since anaphylactic sensitization resulted from this treatment.

In the case of experimental ivy sensitization, there are in all probability factors additional to those which operate in the experiments

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[3] We are indebted for this technique to Drs. Austin L. Joyner and Philip D. McMaster.

[4] In the experiments on poison ivy, with animals suitably restrained from activity, the base of the operated area remained dry and there was no indication of seepage from the cut edges.

with proteins, for with poison ivy, despite the use of an excess of ivy left in place on the isolated skin of such islands for several days, we find not merely a decrease in sensitization but in most cases nearly complete inhibition.   That an essential difference exists in the mechanism is indeed known since proteins sensitize by any route while in order to induce skin sensitization towards simple compounds application of the incitant to or into the skin plays a special but probably not entirely mandatory,[5] rôle.   It is of interest that with a substance neither protein nor fat-soluble, salvarsan, sensitization was also seen to be entirely prevented following intradermal injection into an island, and here the resulting sensitization is not of the contact dermatitis type.

Why in the case studied of hypersensitiveness to poison ivy free lymph circulation is a necessity for the sensitization process we are not in a position to say.   The existence of the highly developed lymph system of the skin (15, 9) may come into consideration,[6] and also the altered state of the tissues in the skin island (cf. 8), as evidenced by a rather persistent edema; yet there well may be involved a disturbance of a special mechanism which is still unknown.   A unique importance of the lymph glands themselves cannot be concluded without specific evidence, in view of other immunological knowledge, although the importance of these in the production of certain antibodies was demonstrated in experiments of McMaster et al. (18, 12).

To some extent the special conditions obtaining in the skin seem to play a part in sensitization with common protein antigens also. Thus Sulzberger (19) observed that the minimum amount of horse serum needed for anaphylactic sensitization was smaller when administered by the intradermal than the subcutaneous route, a result which we were able to verify in more extensive tests, although the ratio of the minimum doses varied from one experiment to another.

The production of a local sensitivity by application of ivy to an island has so far not succeeded, and the question may still be open

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[5] Experiments in progress appear to show that a dermal sensitization may be induced by intraperitoneal injection with the aid of adjuvants, e.g. with picryl chloride (vide 14) and killed tubercle bacilli.

[6] As regards peculiarities of the immunological rôle of the skin tissues, see (16, 17).

whether the process of sensitization can be completed in or by the dermal tissues alone; on the other hand, a part of the skin isolated with interruption of the lymphatics can take part in the general sensitivity produced upon treatment of the skin outside, and this is hardly to be explained otherwise than by the intermediacy of the blood stream.

SUMMARY

Experiments are described on the latency period in sensitization to poison ivy and on the time necessary for the agent to remain in contact with the skin. The chief matter of investigation concerned the manner in which the whole skin becomes sensitive following treatment at a particular site, and especially whether this is effected by way of the epidermis.

Two methods were used to interrupt the continuity of the skin, one by cutting through both skin and the underlying thin muscular layer, the other by removing a strip of skin so as to spare the skin muscle. These procedures led to different results when poison ivy extract was applied to the areas thus isolated. In the first case, sensitization was mostly prevented, whereas with the second method generalized hypersensitiveness occurred almost uniformly.

An explanation is to be found in the severance of the lymph vessels lying on the surface of the muscular layer, pointing to the necessity of a free lymph passage. On the other hand the experiments prove that general sensitization is not dependent upon maintaining the integrity of the skin around a treated area.

An inhibition of sensitization by incisions extending through the panniculus carnosus was seen to some extent in anaphylactic sensitization with protein antigens, namely when sufficiently small amounts were employed.

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EXPLANATION OF PLATE 42

FIG. 1. Test for hypersensitiveness on the 14th day after operation (and ivy treatment) as shown in Fig. 3, with two different concentrations of ivy extract.

FIG. 2. Similar test after the operation as shown in Fig. 4.

FIG. 3. Isolated area of skin on the flank, with severance of the panniculus carnosus, after applying poison ivy and dressing.

FIG. 4. Skin island as above, but with panniculus carnosus spared.

FIG. 5. Section across the marginal part of the skin defect made as in Fig. 4. The skin muscle is intact and is covered on the larger (central) part with only a thin layer of loose connective tissue.   ×8.

PLATE 42

1

2

3

4

5

ELECTROPHORESIS OF ANTERIOR PITUITARY PROTEINS*

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The hormones produced by the anterior lobe of the hypophysis
appear to be proteins. Consequently, the chemical methods employed
for the extraction, separation, and study of these hormones are the
methods of protein chemistry. The questions arise as to whether
the preparations thus obtained satisfy the criteria of homogeneity,
whether the various hormones exist in the gland tissue as chemical
individuals or whether they are split off from a "master molecule"
in the course of the preparation. It would seem that these problems
might profitably be attacked by physical-chemical methods of the
type of size analysis by ultracentrifugation and ultrafiltration, and
studies of the behavior in an electrical field. The latter techniques
possess advantages over the more drastic chemical methods.

The present experiments attempt to apply the moving boundary
method of electrophoresis, with optical observation by the Toepler
schlieren method, to this problem. The results afford a first orienta-
tion in a highly complex system composed of a number of physiolog-
ically active and inactive proteins. The data illustrate the applica-
bility of the technique to the study of tissue extracts and of purified
fractions isolated therefrom by chemical methods. In this respect the
present experiments supplement the recent work of Tiselius (1–4) who

has demonstrated the value of electrophoretic analysis in the study of blood sera, serum proteins, and specific antibodies.

Apparatus

The general arrangement of the electrophoretic apparatus employed in the present study is that described by Tiselius (1, 5).   Inasmuch as the apparatus incorporates a number of minor modifications, and in some respects is simpler than the original apparatus of Tiselius, a short description will be given.

The parts composing the optical tract for analysis by the Toepler schlieren method (6) are indicated in Fig. 1 which is a schematic representation of the entire arrangement.   The insulated tank in which the electrophoresis cell is immersed has a capacity of about 100 liters.   It is kept close to 4° by an electrolux gas refrigerating unit (50 kcal./hours capacity) and a circulation system filled with 50 per cent alcohol.   The motor-driven stirrer and the circulation pump are mounted on a Lally column placed on a sheet of rubber packing, thus preventing the transmission of vibrations to the electrophoresis cell.   The light source and the camera are mounted in a similar manner.   The light source is a Bausch and Lomb microscope lamp equipped with a special iris diaphragm and a rack and pinion drive for the first condenser lens (B in Fig. 1).   The schlieren lens, E, is mounted on a sturdy bracket, supported by the table carrying the water tank, and in such a manner as to be very close to the double window of the tank (100 mm. free diameter).   The camera consists of two lengths of heavy walled brass tubing turned to ensure a sliding fit.   The electrophoretic diagrams are recorded on standard 35 mm. Eastman positive film contained in a Leitz eldia printer.   The latter fits tightly into a brass casing mounted on a slide permitting a rapid change from photography to visual observation on a ground glass screen.

The lenses incorporated into the apparatus are corrected for spherical and chromatic aberration, thus eliminating the need for light filters.   Their focal length is not optimal but the main condition for analysis by the schlieren method, *viz.*, that the schlieren lens, E, must reproject the edge of the first schlieren diaphragm (near the light source) into the plane of the second, movable schlieren diaphragm (in front of the camera lens), is fulfilled.   On the other hand, the camera lens is not focused on the U tube of the electrophoresis cell; this results in a ratio of the size of the index to that of the image of approximately 2.5:1.   In actual practice this has not been found to represent a serious drawback, while the lens combination and the distances chosen have made it possible to accommodate the entire apparatus in the space available, *i.e.* an over-all length of 450 cm.

The type of electrophoresis cell employed depends on the aim of the experiments.   For purposes of examination of a given protein preparation for the number

of principal components present, for studying the degree of homogeneity of purified fractions, and for determinations of electrophoretic mobility, the simple U tube cell (here called "analytical cell") described by Tiselius in 1930 (7) may be used to advantage. The cell used in this laboratory consists of pyrex glass and is made of one piece. The U tube index lengths available for observation are rectangular openings of 40 mm. length and of the width of the tubing (8.5 mm.) in the metal shield carrying the electrophoresis cell. The cross-section of the U tube is 0.587
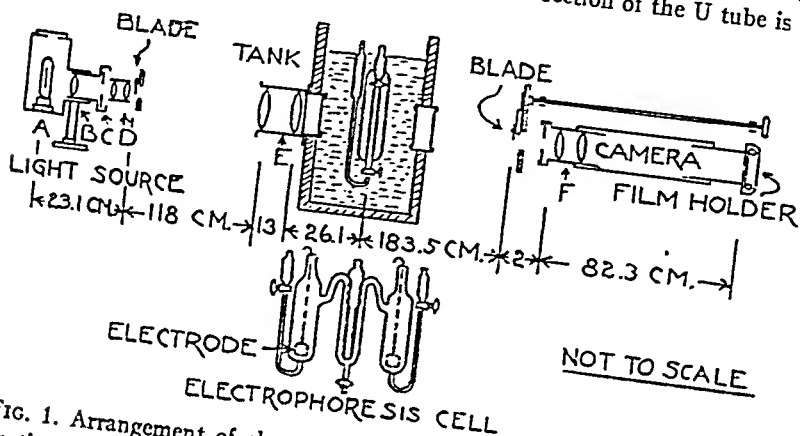
FIG. 1. Arrangement of electrophoresis apparatus (schematized). A, mazda projection bulb, 200 watts, 110 V; B, condenser lens, plano-convex, 60 mm. diameter, 100 mm. focal length; C, iris diaphragm, closed down to about 2 mm. opening; D, condenser system, formed by two plano-convex lenses of 45 mm. diameter, 40 mm. focal length; blade = first schlieren diaphragm, consisting of a circular fixed diaphragm of 7 mm. diameter and of a sharp edge crossing the opening in the upper third; E, schlieren lens, 22 × 20 rectilinear (R. and J. Beck), 95 mm. diameter, 762 mm. focal length, distance between the two component lenses 130 mm.; blade = second schlieren diaphragm, consisting of a fixed circular opening of 28 mm. diameter and of a horizontal sharp edge movable by remote mechanical control and rack and pinion drive across the opening (from above); F, camera lens, 75 mm. diameter, 390 mm. focal length, distance between the two component lenses 75 mm. (Voigtländer).

sq. cm. The current is supplied by dry batteries (radio B batteries). Potentials up to 450 volts and 8 milliamperes have been used in fairly concentrated protein solutions (1 to 2 per cent) without serious convection currents and boundary disturbances if the temperature is kept close to that corresponding to the maximum density of water. It is preferable, however, to apply lower potentials (about 180 volts) and lower current strengths (less than 4 milliamperes) to ensure the absence of such disturbances even in dilute protein solutions. The advantage possessed by this cell over that developed more recently by Tiselius (5) is the simplicity of

its manipulation, except for the process of stratification of the protein solution under the buffer. However, with a little practice the optimal position of the stopcock controlling the flow of the protein solution from the storage vessel into the U tube may be readily duplicated in successive experiments. Furthermore, under the particular conditions prevailing in the apparatus and with the light source employed in the present experiments, the images of the protein boundaries obtained with the simple U tube cell in the light path are more satisfactory than those obtained with the plane-parallel glass compartments of the new electro-phoresis cell of Tiselius. This is due to the fact that the U tube cell, in contrast to the new, rectangular cells, acts as a cylindrical lens and obliterates the structural details of the lamp filament that are brought out by the rectangular new cells. A limited number of experiments have, however, been conducted with the aid of the new electrophoresis cell of Tiselius (5). The object of the latter runs was either to effect a mechanical separation of the various protein fractions after completion of the experiment, or to obtain an increase in depth of solution in order to detect minor protein components in mixtures. When the new electrophoresis cell is used, higher current intensities (up to 15 milliamperes) may be employed than are possible with the cylindrical cell, without causing thermal boundary disturbances. The compensation arrangement consists of a rod of insulating material which is raised out of one of the electrode vessels by a simple clockwork equipped with a multi-step aluminum pulley in place of the hour hand.

Procedure

The majority of the experiments were conducted in the vicinity of pH 8, where the mobility of most proteins is high enough to effect a resolution of mixtures within relatively short periods (about 2 hours), and where all of the proteins of the pituitary gland appear to be sol-uble. The ionic strength of the buffers used was adjusted to be about 0.1. For experiments at pH 8, the preparations were equilibrated through cellophane against a large volume of 0.017 M phosphate (pH 8.0) for about 16 to 18 hours at 2–3°C. Precipitates which formed in some instances were removed by filtration. The outside fluid was employed as the supernatant buffer in the electrophoresis. The hydrogen ion concentration was determined with the glass electrode. The conductivity of the buffer solutions after dialysis was measured with the aid of a Leeds and Northrup bridge arrangement and a Washburn conductivity cell designed for fluids of medium conduc-tivity. This measurement was carried out at the same temperature as that at which the electrophoresis was performed.

The potential gradient was calculated by means of the formula $F = i/q \times \kappa$, where F is the potential gradient in terms of volt per

cm. of the U tube, i the current intensity in amperes, as read from a milliammeter (Triplett universal instrument) connected in series with the electrophoresis cell, q the cross section of the U tube in square centimeters (determined by calibration with mercury), and κ the conductivity of the solution (Tiselius (7)).

The boundaries developing in the course of the electrophoresis were recorded photographically at intervals, but the process was also followed by frequent visual observations, on a ground glass screen, and at various positions of the movable schlieren diaphragm. With the arrangement of the blade employed in the present experiments, the boundaries appear as bright lines on a dark background. For quantitative determinations of the electrophoretic mobility it is necessary to measure the change in position with time of the various protein boundaries with reference to an index line. For this purpose, two sharp lines were imprinted in each exposed field with the aid of a mask placed in the back of the film and an auxiliary light source consisting of a miniature flash light bulb placed in the film camera. For the measurements, the developed film was held flat under spring pressure, and the distance between one of the index lines and a sharp edge of the boundary was determined with a simple measuring microscope (Unicam Company, Cambridge, England) equipped with a cross-hair eye piece and capable of reading to 0.01 mm. These distances were measured in a series of exposures, and the mean of the change with time was calculated. The value thus obtained was multiplied with the photographic reduction factor and reduced to the basis of motion in cm. per second per volt in order to give the absolute mobility, u. The latter was found, in accordance with Tiselius, to be of the order of 10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Pituitary Preparations Studied

Crude Extracts

Burn and Ling Extracts.—These extracts, prepared according to Burn and Ling (8), represent alkaline extracts (pH 11 to 12) of the anterior lobes of fresh beef pituitary glands; the extracts are neutralized to pH 7.5 subsequent to the extraction process. 1 cc. is equivalent to approximately 0.2 gm. of fresh gland tissue. It should be mentioned that the extracts used in the present experiments had been subjected to an additional procedure not ordinarily followed in the

preparation of Burn and Ling extracts, *viz.*, to freezing of the extract after adjusting the pH to 7.5.   This treatment causes the flocculation of a considerable quantity of contaminating substance of gummy consistency.   After centrifuging the thawed solution a comparatively transparent fluid is obtained.   The total protein content of these solutions was found to be approximately 1 per cent.

Glycerol Extract.—50 gm. of anterior lobe tissue of fresh, dissected glands were ground finely with sand and suspended in 50 cc. of glycerol. The suspension was covered with a layer of toluene.   It was then kept at room temperature for 6 days with shaking at intervals.   After adding the same volume of distilled water the mixture was filtered by gravity.   The color of the clear solution was cherry red.   The procedure here employed is somewhat similar to the one ordinarily used for the extraction of endocellular proteolytic enzymes from animal tissues.   The next task was to remove the glycerol which would probably interfere in the electrophoretic experiments by altering the charge and mobility of the protein components (Ågren and Hammarsten (9)).   30 cc. of the glycerol extract were dialyzed against 450 cc. of a 1 per cent NaCl solution at about + 5° for 72 hours; the NaCl solution was changed every 24 hours.   The resulting preparation had a total protein content of 0.4 per cent and was used for electrophoresis.

Saline Pituitary Extracts.—The saline extracts employed were prepared by a procedure developed in this laboratory (10).   Essentially, the extracts are preparations obtained by the extraction of fresh, ground anterior lobes of pituitary glands with either 10 or 2 per cent NaCl solutions at pH 7.0 to 7.5.   The suspensions were centrifuged in a laboratory centrifuge at about 2000 R.P.M. and the turbid supernatant fluid was clarified somewhat by the use of a Sharples supercentrifuge.   The slightly opalescent, reddish extracts thus prepared were found to contain approximately 1 per cent total protein.

Fractions and Purified Extracts

Growth Ketogenic and Prolactin Fractions.—The details of the preparation of these fractions have been described (11).   Essentially, they represent components of a fraction of a Burn and Ling extract which are soluble in 80 per cent acetone and which are obtained by isoelectric

precipitation. The Burn and Ling extract is adjusted to pH 3.5 with HCl and 4 volumes of acetone are then added. The precipitate is discarded and the supernatant solution is treated with one volume of acetone, bringing the total acetone concentration to 90 per cent. The resultant precipitate is dissolved in water and separated into two fractions insoluble at pH 6.8 and 5.5 respectively. The former fraction is high in growth and ketogenic activity and the latter high in lactogenic content. As determined by the pigeon local crop gland method of bioassay (12) the latter fraction exhibits ten times greater lactogenic potency than the Burn and Ling extracts.

Saline Fractions.—

Fraction A.—This fraction represents essentially the precipitate obtained by adjusting the centrifuged 2 per cent saline extracts to pH 5.3, and exhibits a definite crop-proliferating activity (10). This preparation is relatively free of certain of the other types of physiological activity which have been attributed to crude extracts of anterior pituitary tissue; *e.g.*, growth, diabetogenic, and thyrotropic activity.

Fraction B.—This fraction, also obtained by fractionation of the crude saline extract, exerts a growth-stimulating effect when tested in hypophysectomized rats and also possesses a strong "diabetogenic" potency as tested in the partially depancreatized rat (10).

Crystalline Prolactin.—The crystalline prolactin used in the present investigation was obtained by the procedure described by White, Catchpole, and Long (13).

RESULTS

Crude Extracts

Burn and Ling Extracts.—15 experiments were carried out with this type of extract, 5 of these with the "separation chamber" and the remaining 10 with the analytical cell. In the earlier experiments with the analytical cell a number of boundaries (up to 8) were seen. Subsequently, however, only 2 main boundaries could be distinctly observed and photographically recorded. Hence it must be concluded that some of the additional boundaries seen earlier were due to thermal convection currents (false boundaries). The mobilities of the two main proteins were determined in 3 experiments; the values thus ob-

tained were $u = -4.1$ and -8.8×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} respectively at pH 8 (average values). At pH 8.7 the u values were -5.3 and -10.45×10^{-5} (1 experiment). The pH range for conducting the experiments with crude pituitary extracts is limited to the alkaline region because in other pH regions protein precipitates are formed which render impossible the analysis of the total molecular species present.

When the same extracts were subjected to electrophoresis in the new type of separation cell of Tiselius, the greater depth of solution (30 mm. as compared with 8.8 mm. in the analytical cell) permitted the observation of protein components present in smaller concentrations. Under these conditions, there were always observed faint additional boundaries of protein components of a mobility intermediary between that of the two main proteins. Up to 3 such faint boundaries were detected and photographed with certainty, under conditions excluding the possibility of false boundary formation. Inasmuch as prolactin has a mobility intermediary between that of the two main proteins (see below), it is likely that these faint boundaries correspond to protein hormones of the pituitary gland.

In one experiment with the separation cell, using the compensation device (5), the contents of each of the four chambers were recovered after the experiment and bioassays performed on the solutions contained in the right upper anode and in the left upper cathode compartments. The former, according to visual and photographic observations, contained the front boundaries of the fast main protein and of several proteins of intermediary mobility and present in low concentrations, while the latter contained the slow main protein and a small amount of a red pigment (hemoglobin?). The bioassay showed that the solution in the right upper anode compartment had lactogenic activity whereas the solution in the left upper cathode compartment had no lactogenic activity. In addition, thyrotropic and ketogenic activity was demonstrable in both fractions.

Glycerol Extract.—A sample of the extract (previously dialyzed to remove glycerol) was dialyzed against 0.017 M phosphate buffer of pH 8 in the refrigerator. When this solution was subjected to electrophoresis, the photographic record again showed the presence of two main components, while visually, upon suitable adjustment of the movable diaphragm, up to four components could be distinguished.

The mobility of the two main components, one of which was present greatly in excess of the second, was u = $- 7.5 \times 10^{-5}$ for the fast main component and $- 1.48 \times 10^{-5}$ for the slow minor component, at a pH of 7.65. The mobility of the latter was so small that it must have been close to its isoelectric point. While the fast main component may possibly be identical with the fast protein present in the Burn and Ling extracts (see above), this slow component with an isoelectric point close to pH 7 was not found in the Burn and Ling extract. On the other hand, the second main component of the latter extract (u = $- 4 \times 10^{-5}$ at pH 8) was not present in any appreciable amount in the glycerol extract.

Saline Extract.—A crude pituitary extract prepared with the aid of 10 per cent NaCl solution was dialyzed twice against 25 volumes of 0.017 M phosphate buffer (pH 8) and subjected to electrophoresis at pH 7.3. Two main components were recorded on the film with mobilities, u, equal to $- 1.14 \times 10^{-5}$ and $- 4.25 \times 10^{-5}$. The slow component is probably identical with the corresponding protein found in the glycerol extract, while the faster component is probably the same as the slower main component in the Burn and Ling extracts. A saline extract prepared with 2 per cent NaCl showed two boundaries with mobilities 0 and approximately $- 13 \times 10^{-5}$ at pH 7.9.

Purified Fractions

Growth Ketogenic Fraction (GK Fraction from Burn and Ling Extract).—With this fraction, two boundaries were observed in the analytical cell and up to four boundaries in the separation cell. Owing to the solubility properties of this protein mixture, the experiments had to be conducted on the acid side of the isoelectric point. The mobility of the main component at pH 4.86 was $+ 8.99 \times 10^{-5}$.

Prolactin Fraction from Burn and Ling Extract.—Visual observation disclosed as many as four boundaries in the analytical cell. The mobilities of the two main components at pH 8.05 were $- 6.45$ and $- 9.78 \times 10^{-5}$. The latter protein may represent either the fast main protein of the Burn and Ling extract or denatured prolactin (see below), while the protein with a mobility of $- 6.4$ was very probably native prolactin (see below).

Fraction A (from Saline Extract).—A 1 per cent aqueous solution of this fraction was dialyzed against 0.017 M phosphate buffer (pH 8.0).

The pH of the supernatant buffer, after equilibration, was 8.07. Employing a potential gradient of 5.95 volts per cm., only one main component could be detected; the mobility of this component was $- 10 \times 10^{-5}$ at 11°C. The prolactin present did not appear as a separate boundary, presumably because of its low concentration.

Fraction B (from Saline Extract).—A 1 per cent aqueous solution of this fraction was mixed with an equal volume of 0.017 M phosphate buffer (pH 8), and then dialyzed against an additional quantity of the same buffer. After equilibration through the membrane, the pH of the solution was 8.09. The fraction appeared to be relatively homogeneous upon electrophoretic analysis, only one faint boundary being observed visually in addition to the main boundary. The average mobility of the main component at the pH of the experiment was found to be $- 9.8 \times 10^{-5}$ at 3°C.

Crystalline Prolactin

125 mg. crystalline prolactin were dissolved in 25 cc. of 0.017 M phosphate (pH 8) and dialyzed for 24 hours in the ice box against 500 cc. of the same buffer. The first electrophoretic experiment at pH 7.98 and at two different potential gradients (6.55 and 8.3 V/cm.) revealed only one sharp boundary indicative of a high degree of homogeneity of the crystalline protein. The mobility at pH 7.98 was $- 6.4 \times 10^{-5}$, *i.e.*, intermediary between the mobilities of the two main proteins of the Burn and Ling extracts. After the experiment the prolactin solution was recovered from the electrophoresis cell and stored for 19 days in the frozen state. In order to shift the pH of the solution, the material was thawed and dialyzed overnight in the refrigerator against 500 cc. of acetate buffer, pH 3.9 (0.02 N in sodium acetate and 0.1 N in acetic acid). After equilibration, the pH of the outside fluid, used as the supernatant buffer in the electrophoresis, was 3.94. The material again appeared perfectly homogeneous at a potential gradient of 7.06 V/cm. The mobility at pH 3.94 was found to be $+ 9.61 \times 10^{-5}$.

The solution in acetate buffer was again frozen and stored for 2 days. After thawing it was dialyzed overnight against 500 cc. of 0.03 M phosphate buffer of pH 6.5 (ionic strength approximately 0.05). After 20 hours' dialysis there was a heavy precipitate of protein in the dialyzing bag; the pH of the outside buffer was 6.0. The

TABLE I

Electrophoresis of Pituitary Preparations

Experiment No.	Type of extract or fraction	pH	Potential gradient	Number of components	Mobility $u \times 10^5$ [+ and − refer to the charge on the protein]	Temperature†	Notes‡
			V/cm.		cm.² sec.⁻¹ volt⁻¹	°C.	
5	Burn and Ling (crude)	7.19		3		23	
8	" " " "	8		2		8	
18	" " " "	8	6.7	2	−4.45; −8.8	2.5	
22	" " " "	7.86	9.4	2	−3.35; −8.75	4	
23	" " " "	7.94	9.0	2	−4.6 ; −8.75	3	
26	" " " "	8		2*		4	Separation cell
27	" " " "	8		4		4	" "
28	" " " "	8		3		4.5	" "
30	" " " "	8		3	.	3	" "
31	" " " "	8		6*		3	" "
34	" " " "	8.67	7.9	2–3	−5.30; −10.45	4	
4	Burn and Ling growth-ketogenic fraction	4.98		2–3		22	
36	" "	4.86	7.85	2	+8.99 (main component)	3.5	
37	" "	4.8	12	2–3		2.2	Separation cell
40	" "	4.8		2–4		6	" "
47	Burn and Ling lactogenic fraction	8.05	6.42	2*	−6.45; −9.78	4	
50	Glycerol extract	7.65	6.4	4*	−1.48; −7.52	2	
54	10 per cent NaCl extract (crude)	7.50	3.61	3*		2–3	
55		7.30	5.66	2	−1.14; −4.25	4	
65	2 per cent NaCl extract (crude)	7.9	4.64	2	0; −13.8	7.5	
67		7.65	2.12	2	0; −18	11	
66	Lactogenic fraction (A)	8.07	5.95	1	Approx. −10	11	Probably "ballast protein"
58	Ketogenic fraction (B)	8.09	5.6	1	−9.8	3	
64	" " "	7.9	6.14	1	−10.6	9	
35	Crystalline prolactin	7.98	6.55 8.3	1	−6.4	4	
46	" "	3.94	7.06	1	+9.61	5	
48	" "	6.52	6.43	2	−3.64; −6.43	4	Aged solution
49	" "	7.72	6.5	2	−5.42; −9.18	3	" "

* The starred values in the column "Number of components" are minimum numbers.

† Only the mobility values obtained at the same temperature are directly comparable. Mobilities obtained at different temperatures must be corrected for the change in viscosity with temperature before comparisons may be made.

‡ All experiments, except those otherwise designated, were carried out in the cylindrical electrophoresis cell of Tiselius (7).
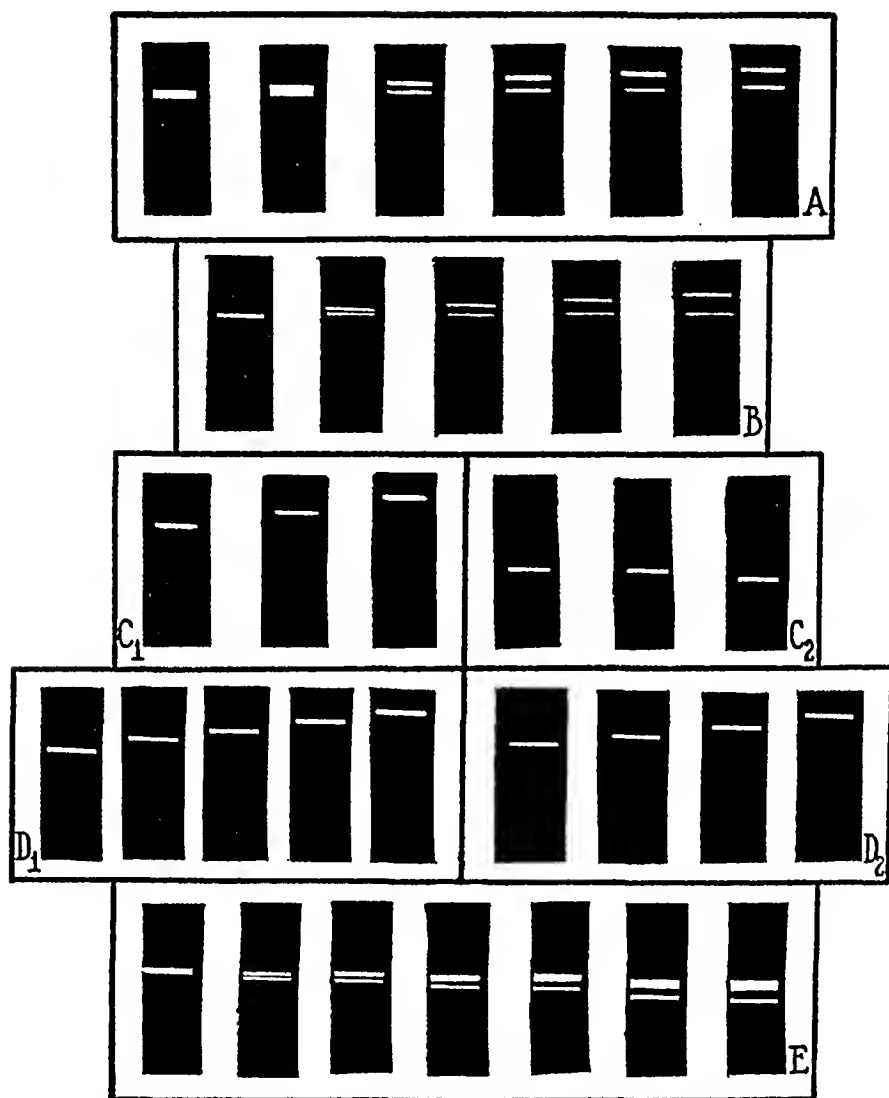
FIG. 2. Toepler schlieren photographs of pituitary preparations in the course of electrophoresis.

A. Crude pituitary extract (Burn and Ling) (Experiment 22). Anode chamber; pH 7.86; $F = 9.4$ V/cm.; 4°C. The two boundaries photographed belong to ballast proteins (mobilities $u = -3.35$ and -8.75×10^{-5}). Exposures (10 seconds) on positive film at intervals of 5 minutes.

B. Crude glycerol extract (Experiment 50). Anode chamber; pH 7.65; $F = 6.4$ V/cm.; 2°C. The two boundaries correspond to ballast proteins (mobilities $u = -1.48$ and -7.52×10^{-5}). Photographs taken at intervals of 10 minutes.

C. Growth ketogenic fraction from Burn and Ling extract (Experiment 36). pH 4.86; $F = 7.85$ V/cm.; 3.5°C. The boundary belongs to the main protein

precipitate was dissolved by adding about 1.5 cc. of 0.5 N KOH to the suspension. The solution was now dialyzed against a fresh portion (500 cc.) of phosphate (pH 6.5). The solution gradually became opalescent during the dialysis, the opalescence persisting upon filtration. The pH of the protein solution after dialysis was 6.55 and that of the outside buffer 6.50. Electrophoresis at this pH now revealed the presence of two proteins in the solution with sharp boundaries, the mobilities being -3.64 and -6.43×10^{-5} respectively. In order to decide whether the appearance of the second boundary was due to a reversible pH dissociation or to an irreversible decomposition or denaturation of the prolactin in the course of the preceding treatment, the solution was again recovered and the pH shifted back in the vicinity of the pH of the first experiment by dialyzing against 500 cc. of phosphate solution (pH 8). The pH of the supernatant buffer was then 7.72. Electrophoresis at this hydrogen ion concentration and at a potential gradient of 6.5 V/cm. showed that the two separate boundaries persisted, $i.e.$ that the change suffered by the protein was irreversible. The mobility of the two components was -5.42 and -9.18×10^{-5}. A plot of the data obtained with the prolactin preparation indicates an isoelectric point of native prolactin at approximately pH 5.6 and it shows that one of the two components present in the changed prolactin solution is apparently still native prolactin while the other has a considerably higher mobility and probably rep-

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component of the preparation ($u = +8.99 \times 10^{-5}$). Upon raising the schlieren diaphragm an additional faint boundary of a protein with a smaller mobility could be detected. C_1, cathode chamber; C_2, anode chamber. Interval between exposures, 15 minutes.

D. Crystalline prolactin (Experiment 46). pH 3.94; $F = 7.06$ V/cm.; 5°C. The boundary belongs to the native protein hormone (mobility $u = +9.61 \times 10^{-5}$). D_1, cathode chamber in forward run. D_2, cathode chamber in backward run. Interval between exposures, 10 minutes.

E. Crystalline prolactin (Experiment 48). pH 6.52; $F = 6.4$ V/cm.; 4°C. This solution had been stored previous to the experiment in frozen state at pH 3.94 for 3 days and had been dialyzed (after thawing) against phosphate buffer, pH 6.5, for 48 hours in the refrigerator. The slow major component shows the mobility of native prolactin ($u = -3.64 \times 10^{-5}$), whereas the fast minor component (mobility $u = -6.43 \times 10^{-5}$) corresponds probably to denatured prolactin. Cathode chamber. Interval between exposures, 10 minutes.

resents denatured prolactin. After the last experiment the total protein was recovered by acetone-ether precipitation and it was assayed. The lactogenic activity showed no significant decrease when compared with the original crystalline material.

Some of the results obtained in the present investigation are given in Table I. In Fig. 2 will be found representative photographic records obtained in typical experiments.

DISCUSSION

The bulk of the protein present in crude pituitary extracts consists of physiologically inert material which is probably more or less identical with that encountered in any other type of tissue. The two main boundaries observed in the electrophoresis of such crude preparations are those of the inert or "ballast" proteins. The widely differing electrophoretic mobility of these proteins as observed in extracts prepared by different solvents, e.g., dilute alkali, glycerol, and saline, is an expression of chemical differences between the ballast proteins thus obtained. In other words, each solvent attacks the complex system of protoplasm in a different manner and exhibits a pronounced selectivity with regard to the tissue proteins which it will bring into homogeneous solution. In addition, it is to be expected that treatment at pH about 10 or above, as it takes place in the preparation of the Burn and Ling extracts, may cause irreversible changes in certain of the proteins. The mobility values obtained with proteins subjected to such drastic procedures may conceivably be quite different from those typical for these proteins in their native state. In any event, the determination of the mobility of any protein contained in the gland extracts serves to label this component and allows one to gauge the success of subsequent fractionation procedures.

In a layer of thickness of the crude extracts as it exists in the new rectangular compartments of the Tiselius separation apparatus, several faint boundaries of an intermediary mobility were detected. There is reason to believe that the proteins responsible for these additional boundaries represent, at least in part, pituitary hormones. This is concluded from the fact that crystalline prolactin exhibits a mobility of the same magnitude as one of the minor boundaries seen in crude Burn and Ling extracts.

A fraction with high lactogenic activity, obtained from a Bum and Ling extract, showed two main boundaries, one corresponding to one of the ballast proteins of the crude extract and the other corresponding to that shown by crystalline prolactin. It follows that at least one major ballast protein present in the crude extract has been removed by the isoelectric purification procedure employed.

Freshly prepared solutions of crystalline prolactin yield only a single boundary. The high definition and small rate of spreading of this boundary in the course of the migration indicate that the product is, at least electrophoretically, quite homogeneous. The second boundary observed in solutions after storage at low temperature shows a higher mobility and probably corresponds to denatured prolactin. Thus the technique makes it possible to follow the gradual transformation of a native into a partially denatured protein. Other examples of the usefulness of the method will be found upon inspection of Table I.

It would be of importance to characterize the pituitary proteins not only by means of their electrochemical behavior but also by their particle size. A first attempt in that direction has recently been undertaken by Severinghaus and his collaborators (14). The main purpose of this work, however, was "to test the possibility of concentrating and separating the anterior lobe and pituitary-like hormones by means of the ultracentrifuge." Consequently, an air-driven quantity centrifuge (15) instead of an analytical centrifuge was employed in that research. It was found that gonadotropic fractions from pregnant mare serum and from castrate urine could be concentrated by subjecting them to ultracentrifugal fields of 150,000 to 200,000 times gravity for 4 to 6 hours. On the other hand, little if any concentration of a flavianate extract of beef hypophysis, rich in thyrotropic activity, could be achieved by the same procedure. These results suggest that the thyrotropic activity of the pituitary gland is associated with molecules of a size smaller than that of the gonadotropic principle.

It may be stated on the whole, that the application of ultracentrifugal and electrophoretic methods to the study of the pituitary hormones holds considerable promise of enlarging the present knowledge of these important biocatalysts.

SUMMARY

The moving boundary method of electrophoresis has been applied to a study of pituitary extracts and purified protein fractions derived from these extracts. The technique employed was that developed by Tiselius and involved the optical observation of the protein boundaries by Toepler's schlieren method. The present experiments were designed primarily to determine the number of proteins present, the degree of homogeneity of the various fractions, and the electrophoretic mobility of the individual components under standard conditions.

The preparations studied included crude gland extracts obtained with dilute alkali, glycerol, and saline; purified pituitary fractions prepared by isoelectric and precipitation procedures; and freshly prepared and aged solutions of crystalline prolactin.

The bulk of the crude gland extracts is composed of physiologically inert proteins, the gradual removal of which in the course of the chemical purification procedures could be controlled by electrophoretic analysis. Freshly prepared solutions of crystalline prolactin exhibit a high degree of electrochemical homogeneity. Upon storage, however, a second component, presumably denatured prolactin, is formed.

The progress of the work was greatly aided by the interest of Dr. C. N. H. Long.

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EPIDEMIOLOGY OF LYMPHOCYTIC CHORIOMENINGITIS IN A MOUSE STOCK OBSERVED FOR FOUR YEARS

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Choriomeningitis in a mouse colony is a favorable material for the study of the problem of the effect on a parasite of long association with a given host. The host animals can be easily maintained in large numbers in a relatively small space, and two or three generations can be secured in a year, so that progress is rapid for this type of work. A colony of infected mice has now been observed for 4 years and it is the purpose of this report to describe the changes that have taken place in the disease and its causative agent since the epidemiology was first studied (1).

Methods and Materials

The infected stock, which has been kept in a large metal breeding cage, consisted on the average of 12 to 15 mature females, 2 to 3 mature males, 10 to 20 mice just weaned, and a varying number (usually 1 to 4) of litters of suckling mice. The breeding mice selected from as many different litters as possible were kept for about 7 months and then replaced by animals that had just become sexually mature. Young mice not needed as replacements were discarded at the age of about 4 weeks. The mice removed from the colony were tested for circulating virus from time to time in order to be certain that the disease was still present. Cannibalism occasionally caused losses among the suckling mice. Fighting between the males has been rare. When it occurred the most aggressive animal was removed and replaced by an immature one.

The fertility in the infected stock was considerably below that in the virus-free colony derived from the original infected stock late in 1934 (1). This fact, however, can hardly be attributed to the disease alone, since it has been found that mice breed less regularly when a large number are kept in one cage. In the virus-free colony 1 male and 4 females are kept together in a smaller cage, and under such conditions the mice breed very regularly.

The virus-free mouse stock just mentioned was built up on 6 uninfected mice

(3 males and 3 females) from the mouse colony in which choriomeningitis was discovered in 1934. The progeny of these 3 pairs of mice were carefully tested for the absence of the infection and then cross-bred. This new stock of mice has since remained free from the disease. The mice are uniformly susceptible to choriomeningitis and there is no evidence that their susceptibility has changed since 1935, when the first epidemiological experiments were performed. The health and general condition of the colony are very good.

The infected and the virus-free mouse stocks have been quarantined as strictly as possible. No new mice were added to the colonies, and the diet as well as the environmental conditions was not changed until May, 1938, when the infected stock was taken to Germany.

Further technical details will be given in the text.

A Change in the Mode of Transmission of the Disease

Two modes of transmission of the virus from mouse to mouse were observed in 1935, intrauterine and contact infection. Pregnant females that continued to carry virus in the blood after clinical recovery often transmitted the virus to their embryos. Other mice which did not become infected *in utero*, contracted the disease by contact soon after they were born.

Choriomeningitis was essentially a disease of young mice of which many became severely ill and some died during the first month of life. Other mice contracted a subclinical infection. In the period between October 7 and December 11, 1935, for instance, the rate of infection was practically 100 per cent, the morbidity amounted to about 20 per cent, and the mortality to 4.4 per cent in terms of the total mouse population, which then numbered some 150 animals. If the rates of morbidity and mortality had been expressed in terms of the number of immature mice present in the colony, they would have been considerably higher.

The mice infected *in utero* were the only ones to show definite symptoms, while the animals infected by contact shortly after birth merely showed a slightly decreased growth rate. They looked like normal mice to any one not familiar with the exceptionally rapid growth and the large size of the strain of mice used. These observations are based upon the results of a large number of careful tests.

When the epidemiological studies were resumed in 1937, intrauterine infection had become the only mode of transmission of the virus.

This was probably due to the fact that all of the stock mice, young and old, were carriers of virus. A life-long infection was demonstrated in a number of cases, while the other animals were discarded before the duration of their infection could be determined.

A Decrease in the Severity of the Disease and Its Possible Causes

The most striking change that occurred gradually between 1935 and 1937 was a marked decrease in the severity of the disease. Since it is now subclinical, infected young mice can no longer be distinguished with certainty from uninfected ones in spite of the fact that their tissues contain about the same amounts of virus as before the change occurred.

In view of the fact that certain other virus diseases sometimes change in a similar manner, an attempt was made to determine the cause for the present mildness of the disease. Such an attempt was possible because the mice from the virus-free colony, which had been used for intrauterine infection with stock virus in 1935, were available for comparative experiments on intrauterine infection with virus freshly isolated from the infected stock. On the basis of numerous other observations it could be assumed that the susceptibility of these mice had not appreciably changed since 1935, when intrauterine infection produced a severe disease in the majority of them.

The brains, thoracic and abdominal organs of suckling mice from the infected stock were used as a source of virus. The females needed for the experiments were selected from 5 different litters of mice obtained from the virus-free colony and injected intranasally with stock virus at the age of 1 day. Mice infected in this manner carry virus in the blood for a very long time after clinical recovery (2). 9 females whose blood had been previously tested for virus were bred to 9 virus-free males. 2 young from each of the 9 litters obtained were sacrificed immediately after birth for a test for intrauterine infection. Their brains were removed aseptically and each suspended in 2 cc. saline. The brain suspensions were inoculated intracerebrally into virus-free mice in amounts of 0.05 and 0.05 × 10⁻³ cc. If these mice, particularly those receiving the higher dilution, developed the disease, it was assumed that the respective litters had become infected *in utero*. This assumption is justified by previous tests which showed that the virus is transmitted either to all of the embryos or not at all. The litters were watched daily for 2 months.

5 of the 9 litters became infected *in utero*, while the other 4 litters did not in spite of the fact that the mothers still carried the virus in the blood 2 weeks after

parturition.   This fact suggests that the virus content of the blood may not have
been alone responsible for the infection of the embryos of the other mice.   The
absence of detectable amounts of virus from the blood of another naturally in-
fected mouse that transmitted the virus to its embryos (1, Table III) points in
the same direction.   It is possible therefore that the transmission of the virus
takes place either in the ovaries, which contained relatively large amounts of
virus in 2 carriers tested (2), or in the uterus, which likewise may be rich in virus
(2), by "growth" through the placenta.   The term "intrauterine infection" will
be used for the sake of brevity, although it is realized that it may not always be
accurate.

TABLE I

Intrauterine Infection in Mice from the Virus-Free Stock

Litter No.	Date of intra-nasal injection of mother at age of 1 day	Date of birth of litter	Number of young	Result
	1937	*1938*		
1	Oct. 20	Jan. 22	6	1 young became sick and recovered; the others showed decreased growth during the first 2 weeks of life
2	" 26	" 23	7	1 young became sick and recovered; another showed a markedly retarded growth for 4 weeks; the remainder developed normally
3	May 5	" 29	6	No symptoms, but slightly decreased rate of growth
4	Oct. 20	Feb. 1	3	1 young died on the 16th day; the others developed normally
5	" 20	" 22	9	2 young died on the 17th and 37th days; another became sick and recovered; the remainder showed no reaction

Table I shows that the majority of the mice from the 5 litters in-
fected *in utero* failed to become sick.   A few of the young however did
show symptoms of the disease and 3 of them died.   On the whole, the
reaction of the animals was intermediate between the severe disease
observed in 1935 and the extremely mild infection seen more recently.
    The interpretation of this result is not easy.   If one assumes that
the susceptibility of the virus-free mice is still the same as in 1935, it
seems that the pathogenicity of the virus for embryonic mouse tissue
has markedly decreased since then.   On the other hand, since the
disease described in Table I was definitely more severe than that now
prevailing in the infected stock, it may be that the mice from the latter

stock now show a higher degree of resistance to intrauterine infection than those from the virus-free colony. In 1935 the susceptibility to intrauterine infection of both kinds of mice was about the same.

The decrease in the severity of the disease resulting from intrauterine infection has been associated with an increase of the virulence of the virus for suckling mice infected by contact shortly after birth, as shown by experiments reported before (2) as well as later in this paper, whereas contact infection in mice older than 2 to 3 weeks is still subclinical as it was in 1935. It would have been of interest to study the effect of contact infection with the stock strain not only in young mice from the virus-free colony, but also in virus-free young from the infected stock. This has not been possible, however, since no mice of the latter type were found.

A Change in the Contagiousness of the Experimental Disease

Experiments on contact infection carried out in 1935 (1, and unpublished experiments) showed that the experimental disease was transmitted by mice of different ages, provided the period of exposure was sufficiently long. Since the disease induced by contact infection was subclinical, it had to be demonstrated by testing the blood for virus or by tests of immunity several weeks after exposure. When the experiments were resumed in 1938, different results were obtained in that the infection very rarely passed from mature mice infected experimentally to normal ones. Suckling mice infected by intranasal instillation of stock virus, however, still transmitted the virus to normal mice during the acute and chronic stages of the disease. Mice infected naturally, young ones as well as old carriers of virus, could likewise transmit the infection. To illustrate these observations some recent experiments will be given here in detail.

The mice infected experimentally as well as the uninfected animals exposed to infected ones were obtained from the virus-free breeding colony, while the naturally infected mice came from the infected stock. As in previous experiments, the mature mice used were all females in order to eliminate the possible sexual transmission of the disease, or the infection by biting in males.

The experiments were conducted as follows: Mice infected naturally or experimentally as indicated in the tables were placed in the same cage with some uninfected animals on the 3rd or 4th day after inoculation or removal from the infected stock. Care was taken that the mice which died from the disease were not de-

voured by their cage mates, since we have recently been able to infect 1 of 6 mice by feeding with virulent mouse brain given on bread. Unless otherwise stated, the animals were kept together for 4 to 5 weeks, after which time they were tested for immunity by intracerebral inoculation with highly virulent virus. The animals that failed to show any reaction after the test of immunity were assumed to have become infected by contact, while the mice that showed characteristic symptoms or died were counted as negative. If the injected mice, to which the normal animals were exposed had shown no signs of illness, for instance, after intranasal or subcutaneous injection with virus, they were also tested for immunity at the same time as the exposed mice to make sure that they had become infected. Control mice of about the same age as the tested animals were included in each immunity test. About 90 per cent of these died, while the remainder developed typical, non-fatal choriomeningitis with characteristic tremors and convulsions. There was not a single control mouse that failed to become sick.

Exposure of Normal Mice to Mature Mice Infected Experimentally.— The details of these experiments are recorded in Table II which shows that the 5-week-old mice usually failed to transmit the infection, no matter by which route they were inoculated.

Since mice injected intracerebrally with virus often do not survive for the period of time that would be necessary for the transmission of the disease (1), the 6 animals injected in this manner with small amounts of virus in Experiment 1 were each given 0.25 cc. hyperimmune guinea pig serum intravenously 3 hours before the virus inoculation. While such serum treatment usually does not prevent the disease, it often renders it non-fatal. Virus was demonstrated in the nasal washings but not in the urine of some serum-treated mice that were sick and ultimately recovered. The mice used in Experiment 4 received no immune serum. They were still sick but evidently recovering when placed in contact with a litter of normal mice on the 9th day after inoculation.

The Influence of the Age at the Time of Inoculation on the Ability of Mice to Transmit the Disease.—The fact that the mothers of litters from the virus-free colony which had been inoculated intranasally with virus at the age of 1 to 7 days always became immune suggested that young mice infected experimentally would transmit the disease more readily than mature mice. That this was the case is shown in Table III.

Mice injected intranasally with virus at the age of 1 day continue to transmit the disease as they grow up in spite of the fact that they show symptoms for only 3 to 4 weeks. This is shown by the experiment recorded in Table IV which was made with 2 mice left over from

the 4th experiment of Table III.   They were sick for about 3 weeks, recovered, and appeared quite healthy at the age of 58 days when they

TABLE II

Exposure of Normal Mice to 5- to 6-Week-Old Mice Infected Experimentally with Stock Virus

Experiment No.	Injected mice				Exposed mice	
	Number of mice	Route of inoculation	Reaction		Age at time of exposure	Number of mice infected as evidenced by acquired immunity
1	10	ip*	4 died; 6 became very sick and recovered		5 wks.	1/8†
	4	sc	None; immunized		5 "	0/4
	6	Immune guinea pig serum iv, virus ic	3 died; 3 became sick and recovered		5 "	0/4
2	6	iv	4 died; 2 became very sick and recovered‡		5 "	0/7
3	3	iv	Exposed on 13th day after inoculation when 2 mice had recovered and the 3rd still appeared sick		5 "	0/7
4	2	ic	Just recovering from typical disease		1 day (4–5 mos., mother)	0/5 0/1
5	4	iv	Very slight symptoms followed by quick recovery		1 day (4–5 mos., mother)	0/10 0/1

* ip = intraperitoneally.   sc = subcutaneously.   iv = intravenously. ic = intracerebrally.

† 1 out of 8 mice became infected.

‡ The 2 survivors still discharged virus with the urine and nasal secretions at the end of the period of exposure.

were exposed to a newborn normal litter of mice to which they promptly transmitted the disease.   At the age of 108 days the 2 mice were again exposed to normal mice of different ages, but for a shorter

TABLE III

The Influence of Age at the Time of Intranasal Infection on the Transmission of the Disease by Contact

Injected mice			Exposed mice	
Age at time of inoculation	Number of mice	Reaction	Age at time of exposure	Number of mice infected as evidenced by disease (in young mice) and acquired immunity
1 day	5	Slight illness; recovery	1 day	5/5
1 "	10	1 died; 5 became sick and recovered; 4 showed no definite symptoms	1 " Full grown mother	8/8 1/1
1 "	8	4 died; 4 showed retarded growth	1 day Full grown mothers 5 wks.	10/10 2/2 8/8
1 "	7*	2 died; 2 became sick and recovered; 3 showed only a retarded growth	5 "	7/7 (Mother of litter injected intranasally also became immune)
2–3 wks.	4	2 became sick and recovered; 2 showed no symptoms	2–3 "	0/6
2–3 "	3	1 became very sick and recovered; 2 showed no definite symptoms	5 "	1/5
5 "	5	None; immunized	1 day Full grown mother 5 wks.	0/7 0/1 0/8
5 "	6	1 became sick and recovered; 5 showed no symptoms; all became immune	1 day Full grown mothers	0/8 0/2
5 "	6	" "	5 wks.	0/8

* 2 females of this group were used in the experiment recorded in Table IV.

period of time.   The disease passed to the majority of the young mice but not to the older ones.   This result suggests that young mice contract the infection more readily than mature ones and confirms a previous observation (1).

Infection of Normal Mice by Exposure to Virus Carriers.—That full grown mice from the infected stock, which continue to carry virus in the blood and discharge it with the urine and nasal secretions, can

TABLE IV

Continued Transmission of the Infection after Recovery by Two Mice* Injected Intranasally with Virus at the Age of 1 Day

Age of 2 infected mice when placed in contact with normal mice	Exposed mice		
	Age at time of exposure	Period of exposure and method of testing for infection	Number of mice that became infected by contact
58 days	1 day	Tested for virus in blood on 19th day of exposure	5/5 (All young sick when tested)
	Full grown mother of this litter	Tested for immunity on 25th day of exposure	1/1 (Showed no symptoms)
108 "	1 day	Exposed for 13 days; tested for immunity 2 wks. later	6/8 (The infected mice had shown symptoms)
	5 wks.	" "	0/8

* See footnote to Table III.

transmit the disease to healthy mice has already been reported.   This still is the rule, as Table V shows.   The disease readily passes from carriers to normal mice of different ages.   The majority of the carriers used in these experiments came from litters infected *in utero* that were used in previous experiments (2, Text-fig. 1).   They looked quite healthy and could not be distinguished from normal animals.

Comparative Experiments on Contact Infection with Naturally Infected Carriers and Mature Mice Infected Experimentally.—In the following experiments an attempt was made to determine why the disease is often transmitted by healthy appearing carriers but rarely by mature mice infected experimentally.   It was not unlikely that the virus content of the nasal secretions, which appears to be more important

for the transmission of the disease than the urine (1), had some connection with this discrepancy. To test this possibility the virus content of the nasal washings from mice infected either naturally or experimentally was determined before and after exposure to normal animals. Each infected female mouse was placed in the same cage with 5 virus-free 5-week-old females, which were tested for acquired immunity after an exposure for 32 days.

TABLE V

Infection of Mice by Exposure to Old Carriers

Old female carriers infected *in utero* to which normal mice were exposed	Age of carriers at time of exposure	Exposed mice			
		Number	Age when exposed	Number of mice that	
				Showed symptoms	Became immune
	mos.				
2 of Litter C*	10	4†	1 day	2	4
		Mother	4–5 mos.	0	?‡
2 " " D*	10	4†	1 day	2	4
		Mother	4–5 mos.	0	1
3 " " B*	10	1♀	4 "	0	1
2 " " "	13	4♀	5 wks.	0	4
2 full grown from infected stock	4–5	4♀	5 "	0	4

* See Text-fig. 1 in a previous paper (2).
† Virus demonstrated in pooled blood from each group of suckling mice on 19th day of exposure. Mothers not tested for circulating virus.
‡ Death from injury by intracerebral test inoculation.

The nasal washings were taken as already described (2) and tenfold dilutions of them were made in saline. These as well as the undiluted materials were inoculated subcutaneously in amounts of 0.5 cc. into 5-week-old mice from the virus-free stock, one mouse being used for each dilution. It was not practical to titrate the nasal washings by intracerebral inoculation because of the bacteria ordinarily present in them. These were without effect when injected subcutaneously. Since mice inoculated subcutaneously with choriomeningitis virus never show symptoms, the number of infected mice was determined by intracerebral immunity tests made 2 weeks after inoculation.

TABLE VI

The Transmissibility of the Disease by Contact in Relation to Virus Content of Nasal Washings

Experiment No.	Infected mice (♀)				Number of exposed mice (5-week-old ♀) infected as evidenced by acquired immunity
	No.	Mode of infection	Titration of nasal washings		
			Before exposure	After exposure	
1	1	Healthy appearing mouse infected *in utero* and carrying virus in blood for over 1 year	10^{-1} i* 10^{-2} i 10^{-3} i 10^{-4} ni* 10^{-5} ni	10^{0} i 10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} i	5/5
	2	" "	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} ni 10^{-5} ni	10^{0} i 10^{-1} ?† 10^{-2} i 10^{-3} i 10^{-4} i	3/4†
	3	Mouse injected iv with stock strain 9 days previously. Still sick but recovering when exposed on 9th day	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} ni 10^{-5} ni	10^{0} i 10^{-1} ni 10^{-2} ni 10^{-3} ni 10^{-4} ni	1/5
	4	" "	10^{-1} i 10^{-2} ni 10^{-3} i 10^{-4} ni 10^{-5} ni	10^{0} ni 10^{-1} ni 10^{-2} ni 10^{-3} ni 10^{-4} ni	0/5
2	5	Healthy appearing carrier from infected stock, 4–5 mos. of age	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} ni 10^{-5} ni	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} ni 10^{-5} i	4/4†
	6	" "	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} i 10^{-5} ni	10^{-1} i 10^{-2} i 10^{-3} i 10^{-4} ni 10^{-5} ni	1/5
	7	Same as Nos. 3 and 4	10^{-1} i 10^{-2} ni 10^{-3} ni 10^{-4} ni 10^{-5} ni	10^{-1} i 10^{-2} ni 10^{-3} ni 10^{-4} ni 10^{-5} ni	0/5
	8	" " " " " "	10^{-1} i 10^{-2} i 10^{-3} ni 10^{-4} ni 10^{-5} ni	10^{-1} ni 10^{-2} ni 10^{-3} ni 10^{-4} ?† 10^{-5} ni	0/5

* i = immunized; ni = not immunized.   † Mouse died from injury after immunity test.

The details of the experiment are given in Table VI which shows that naturally infected mice (Nos. 1, 2, 5, and 6) in general discharged large amounts of virus over a longer period of time than the other animals infected experimentally (Nos. 3, 4, 7, and 8). This may be the reason why the former mice transmitted the disease more readily than the latter animals.

The urine of the infected mice, which may also play a minor rôle in the transmission of the disease, was not titrated because it was often impossible to obtain more than a few drops of it, and these would not have been sufficient for exact titrations. In other experiments, however, the virus content of the urine often ran parallel with that of the nasal washings, and the same may have been the case in the present tests.

Influence of the Strain of Virus on the Communicability of the Disease. —Since the change in the communicability of the experimental disease may have been due to a change of the virus, it was decided to test this possibility in the following series of experiments. Unfortunately it was not possible to compare the stock virus of 1935 with that of 1938 under the same experimental conditions, because we have not succeeded as yet in preserving choriomeningitis virus in mouse tissue for several years without resorting to animal passage. The latter may markedly alter some characteristics of the virus. In fact, no more than 8 intracerebral passages in mice were necessary to change the pathogenicity for guinea pigs of passage strain B (3). Its virulence for mice likewise differs from that of the stock virus (2). In the following experiments the communicability of the infection induced in mice by the passage strain will be compared with that of virus freshly isolated from the infected stock.

When strain B was isolated from a naturally infected stock mouse in 1935 it produced a contagious disease in 5- to 6-week-old mice. This statement is made with some reserve, however, because the number of mice tested was rather small. In one experiment made with virus from the 1st intracerebral mouse passage, 4 mice from the virus-free colony were inoculated intraperitoneally with virus and exposed to 8 uninfected mice for 32 days. The injected animals were ill from the 6th to the 10th days after inoculation and then recovered. Of the 8 exposed mice 6 became resistant to intracerebral inoculation with highly virulent virus.

The virus used in the present experiments had undergone from 30 to 42 passages in 5-week-old mice. The experiments recorded in Table VII were made with 5-week-old females. They are comparable to those given in Table II and therefore need no special description.

TABLE VII

Experiments on Contact Infection with the Mouse Passage Strain in 5-Week-Old Females

Experiment No.	Injected mice				Exposed mice
	Route of inoculation	Number of mice	Reaction		Number of mice infected as evidenced by acquired immunity
1	ic*	1	Dead on 6th day		0/5
	ip	3	None; immunized		
	ic	1	Dead on 6th day		0/5
	sc	3	None; immunized		
	ic	1	Dead on 7th day		0/5
	in	3	None; immunized		
2	Immune guinea pig serum iv, virus ic	5	2 dead on 7th or 8th day; 3 became sick and recovered		0/4
	ip	4	None; immunized		0/4
3	Immune guinea pig serum iv, virus ic	6	3 dead on 7th day; 3 became sick and recovered		0/4
	ip	5	None; immunized		0/4

* ic = intracerebrally.   ip = intraperitoneally.   sc = subcutaneously. in = intranasally.   iv = intravenously.

Their results were uniformly negative and show that the infection with the mouse passage strain in 5-week-old mice is even less contagious than that with the stock strain. A comparison of the results obtained with the mouse passage strain in 1935 and 1937–1938 gives the impression that its communicability has changed in the course of the serial passages in mice.

In the experiment presented in Table VIII newborn mice were used. 2 litters of mice were injected intranasally with the stock strain and 2

others with the mouse passage virus.   On the 4th day after inoculation each group of young together with their uninjected mothers was exposed for 4 weeks to 2 litters of virus-free mice which were tested for immunity at the end of this period by intracerebral inoculation with virus.   It can be seen from Table VIII that the stock strain proved more contagious under such conditions than the mouse passage strain and likewise was more virulent for the mice infected by contact.

TABLE VIII

Comparison of Communicability of Stock and Mouse Passage Virus in Newborn Mice

Strain of virus	Mice injected intranasally with virus				Exposed mice	
	Age at time of inoculation	Number of mice	Reaction		Age at time of exposure	Number of mice infected as evidenced by signs of the disease or acquired immunity
Stock	1 day	18	5 died; 5 became sick and recovered; 4 showed only a retarded growth; the remainder presented no definite signs of illness		1 day	18/18 (9 mice showed symptoms, the others a decreased growth rate)
Mouse passage	1 "	18	11 died; 6 became very sick and recovered; 1 showed no signs of illness		1 "	4/13 (The 4 infected mice showed no signs of disease but were immunized)

Effect of a Change in the Environmental Conditions on the Course of the Epidemic

In May, 1938, a collection of mice from the infected stock together with some mice from the virus-free colony were taken to Germany. The animals were shipped in metal cages with screen covers, and these cages had to be kept close together on the trip.   The fact that the mice failed to become infected is additional proof that the disease is not highly contagious.

The environmental conditions of the mice in Germany differ from those in America, especially as regards climate and diet.   The diet in

America consisted of water, corn, powdered milk, white bread and biscuits, occasionally with lettuce or green alfalfa; whereas the present daily ration comprises water, corn, a special kind of dog biscuit, and rye bread. Green alfalfa is added during warmer weather. Milk is omitted. Both diets appear to be adequate. The method of keeping and handling the animals has not been changed.

The change in the environmental conditions seems to have had no influence whatever on the course and character of the epidemic. The disease is still subclinical. Intrauterine infection appears to be its only mode of transmission, and all of the mice from the infected stock tested have been carriers of virus.

DISCUSSION

The present mildness of the disease in the infected stock appears to have been brought about by a combination of two factors, namely, a change in the mode of transmission of the infection, and a shift in the severity of the disease with regard to the age of the mice at the time of infection. This shift, in turn, seems to have been caused by a decrease in the pathogenicity of the virus for embryonic mouse tissue, and a concurrent increase in the resistance of the stock mice to intrauterine infection. Shifts in the severity of the infection in relation to the age of the host also occur with other virus diseases. Some epidemics of poliomyelitis, for instance, are associated with unusually severe reactions in adults, while the disease in children is milder in contrast to its usual behavior.

If no other shift occurs in the future, one may expect the natural disease to remain mild as long as intrauterine infection represents its only way of transmission. The picture may change, however, when some litters are born virus-free and become infected by contact. In this case one might again find sick mice, unless the resistance to the virus of suckling stock mice has increased also.

The disease has reached a remarkable equilibrium. It no longer causes visible illness, nor is the virus markedly inhibited by the defensive forces of the body. If the virus were a living organism, one might call its present relationship to the host a "perfect parasitism." Theoretically, the mouse is an ideal reservoir host for the virus.

The cause for the change in the communicability of the experimen-

tal disease has not been determined.   Since the contagiousness of the disease seems to depend entirely or in part on the virus content of the nasal secretions, it is not unlikely that in 1935 the virus had a greater affinity for the upper respiratory tract of mature mice from the virus-free colony, which decreased with the progressive adaptation of the virus to embryonic mouse tissue.

Hereditary factors, which may have played a part in the changes of the disease and are considered by some investigators to be of great importance in the epidemiology of infectious diseases, have not been studied, because it was not desirable for other reasons to interfere with the natural course of the epidemic by selective breeding, for instance, by establishing genetically pure mouse stocks.   The possibility therefore exists that the genetic character of the mice has changed since 1935.   In the course of extensive experimental work with mice from the virus-free colony the impression was gained that this stock has not changed genetically.   It is not unlikely, however, that the above mentioned change in the resistance to intrauterine infection of the mice from the infected stock was of a genetic nature.

The immunological factors influencing the disease are fundamentally the same as in 1935.   It has been noted, however, that the number of immune mice whose blood and viscera contained no demonstrable virus progressively decreased in the infected stock.   Today, the very solid immunity demonstrable in all of the stock mice is invariably associated with infection.   Their tissues and blood contain surprisingly large amounts of virus (2).   This "infection immunity" is of the utmost importance for the epidemiology of the disease, because it permits the virus to be transmitted *in utero* with great regularity and no doubt is chiefly responsible for the long persistence of the disease in the infected stock.   It is due to the extremely poor antibody response to the infection in mice as well as to certain other factors which have recently been studied (2).

SUMMARY AND CONCLUSIONS

A small mouse stock in which lymphocytic choriomeningitis is endemic has been observed over a period of 4 years.   The disease has persisted during that time, but it has become so mild that it can no longer be recognized by clinical observation.   In spite of this fact, all of the stock mice tested, both young and old, carried considerable

amounts of virus in their organs and blood. The females readily transmit the infection to their offspring. Intrauterine infection has become the only mode of transmission of the disease in contrast to the situation in 1935 when a certain number of mice were born virus-free and became infected by contact shortly after birth.

The present mildness of the disease appears to be due to two factors, namely, the change in its mode of transmission just mentioned, and a shift in the severity of the disease with regard to the age of the host at the time of infection. This shift has occurred gradually since 1935 when the mice infected *in utero* were the only ones to become sick. Since 1937, however, the virus is quite harmless for such animals and produces symptoms only in suckling mice from the virus-free stock exposed to contact infection. Evidence is presented which suggests that the shift in the severity of the disease was caused by a decrease of the pathogenicity of the virus for embryonic mouse tissue and a concurrent increase of the resistance to intrauterine infection of the mice from the infected stock.

Another change noted concerned the communicability of the experimental disease. In contrast to observations made in 1935 the experimental infection of mature mice from the virus-free colony is now very rarely transmitted by contact to healthy mice, young or old. Suckling mice from the same stock infected by intranasal instillation of virus, however, readily transmit the disease and continue to do so as they grow up. The same is true for mice infected naturally. The reason for this discrepancy has not been ascertained, but it has been shown that naturally infected mice capable of transmitting the disease in general discharge large amounts of virus through the nose for a longer period of time than mature mice infected experimentally which fail to transmit their infection. It may likewise be of significance in this connection that the virus can lose its communicability by animal passage.

A marked change (chiefly climatic and dietary) in the environmental conditions of the infected stock failed to influence the course and character of the epidemic.

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OSMOTIC PRESSURE STUDY OF PROTEIN FRACTIONS IN NORMAL AND IN NEPHROTIC SUBJECTS

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This paper presents studies of protein osmotic pressures aimed to yield evidence on two questions, namely: (1) Whether albumin and globulin in the serum of patients with nephrosis are identical with albumin and globulin in normal serum, and (2) whether the albumin and globulin in the urine of such patients are identical with the corresponding proteins in the serum, either of normal subjects, or of the patients themselves.

1. Differences between Serum Proteins of Normal Subjects and Serum Proteins of Patients with Nephrosis

Earlier studies, reviewed by Goettsch and Reeves (6), and Alving and Mirsky (4), failed to reveal essential differences, either chemical or physicochemical, between normal serum proteins and the proteins in Bright's disease.

More recently, however, evidences of differences have been obtained. Working with the ultracentrifuge, McFarlane (11) has observed an abnormal sedimentation rate with the serum and urine from several cases of proteinuria, including nephritis, which suggested the presence of abnormally polydisperse albumin. As concerns nephrosis in particular, Tuchman and Sobotka (15) have found that the serum albumin contains more tyrosine, the globulin less, than in normal cases; Alving and Mirsky (4) have presented evidence of the existence of an abnormal albumin fraction with a low cystine content; Goettsch and Reeves (6) have observed immunological differences consisting in the fact that nephrotic albumin and globulin fail to precipitate completely with antisera developed against normal albumin and globulin.

Methods

Conditions for Constancy of Protein Osmotic Pressure Measurements —In protein solutions the specific protein osmotic pressure (pressure per unit weight of protein) is constant only at high dilution (1,3). At such dilution the law of van't Hoff relating osmotic pressure to molecular concentration appears to be valid for proteins, since the molecular weights calculated from the pressures agree with those by other physicochemical methods (13). At concentrations over 10 or 20 gm. per liter the specific osmotic pressure of the plasma proteins increases with increasing concentration. The cause of this phenomenon is not entirely certain. Adair and Robinson (3) conclude that Donnan's law does not explain it. The question has been reviewed recently (13). The essential fact is that to yield exact results pressure measurements must be made on solutions dilute enough to avoid the deviation from van't Hoff law.

If serum is diluted enough to make the proteins conform to van't Hoff's law, and if the albumin and globulin fractions have each the same mean molecular size in the serum as in preparations of these proteins separated by salting out methods, the osmotic pressure of the serum should accord with the equation:

$$P = 25\left(\frac{C_a}{W_a} + \frac{C_g}{W_g}\right)$$

(1)

P is the osmotic pressure of the diluted serum in centimeters of water; 25 is the osmotic pressure of a millimolar solution of a non-electrolyte in water at 20°; C_a and C_g represent the concentrations, in mg. per liter, of albumin and globulin, respectively, and W_a and W_g the molecular weights calculated from the specific pressures of separated albumin and globulin. Adair and Robinson (3) have shown the basic validity of this equation by finding good agreement between the osmotic pressures of unfractionated serum at infinite dilution and those calculated from the partial pressures of albumin and globulin, using the molecular weights which they had found with preparations of separated albumin and globulin.

Pressure Measurements.—An apparatus has been recently described (5) which makes the accurate determination of low osmotic pressures a rapid and easy procedure. The amount of protein necessary for

one determination is only 0.2 cc. and its concentration need not be higher than 0.2 per cent. At such low concentrations, the van't Hoff law can be applied without corrections; therefore the results have been expressed, for simplicity's sake, directly as molecular weights.

The procedure previously indicated has been closely followed (5). Except in Table I, nearly all determinations were carried out in duplicate, sometimes triplicate or quadruplicate. There were practically no erratic results. Duplicate determinations usually checked within less than 5 per cent, the mean deviation for all the determinations being less than 2 per cent. The molecular weights given in the tables were calculated with the help of Table I in the preceding publication (5). The lower the concentration, the more closely (excluding experimental errors) should the figures given represent the true molecular weights. Actually, in the range of small concentrations used here, the differences are usually unappreciable.

Preparation of Material.—For the preparation of dilute serum samples (Table II), the blood was simply allowed to clot at room temperature, the cells were discarded after centrifugation, and serum and diluting fluid were mixed in the proportions indicated in Table II. Albumin and globulin concentrations were determined by Howe's precipitation and Van Slyke's manometric micro Kjeldahl method (12).

For the preparation of the protein fractions, it was felt that the simplest procedure would be the best, since it was the least likely to interfere with the state of aggregation of the proteins. In this instance there seemed to be no point in subjecting the material to such drastic treatments as have been applied by some investigators (17), especially since even the most elementary procedure of precipitation has been shown by the ultracentrifuge to cause definite irreversible changes (10), and since our aim for the moment was to find whether there were differences between normal and pathological sera, rather than to isolate more or less artificial products with apparently constant properties.

The procedure, which was entirely conducted at room temperature, was generally as follows:

The blood was allowed to clot and the cells discarded after centrifugation. To about 5 cc. of serum was added an equal volume of saturated ammonium sulfate

solution, and the precipitate formed was filtered off after a few hours. The globulin precipitate was washed several times on the filter with half saturated ammonium sulfate, then scraped from it and dissolved in a little water. Since with nephrotic globulins the solution remained as opaque as milk, it was then in some cases shaken twice with an equal volume of ether, which was syphoned off after centrifugation. This procedure left a practically clear solution, which was transferred into a cellophane bag for dialysis. The albumin was precipitated from the filtrate with an excess of solid ammonium sulfate, filtered off, and then transferred. An alternative procedure, which was successful with nephrotic serum only, was to centrifuge the precipitated albumin. It would then collect rapidly at the top of the tube like a thick yellow paste, and could be scooped up with a spatula, the remaining fluid being water-clear. Apparently the high lipid content of the albumin fraction in nephrosis is responsible for this behavior, since in normal cases centrifugation at usual speed is quite ineffective.

In some cases the procedure was slightly varied. In one case, the serum albumin was caused to crystallize at room temperature by adding M/1 acetic acid to the filtrate from globulin. The crystals were kept in the ice box over 2 months in the mixture recommended by Adair and Robinson (2) before they were dialyzed. In another case, normal globulin scraped from the filter was dissolved in 15 cc. of 0.15 M NaCl, then reprecipitated at half saturation before dialysis. Still in another, normal plasma instead of serum was used for a globulin determination. Details of technique are briefly indicated in the tables.

Dialysis was performed with small sections of cellophane tubing clamped flat against a piece of hard rubber plate and rocked in a trough for a few hours; the outer fluid, 0.15 M NaCl, was often renewed and the gradual decrease in it of ammonia concentration could be easily traced with Nessler's reagent. For the preservation of globulin solutions, a higher concentration was found preferable, therefore concentrated NaCl was added after dialysis so as to make the final salt concentration about 0.9 molar.

It should be clearly understood that the terms albumin and globulin used here mean nothing more than two rather easily separable fractions, and that no claim is made as to their individuality or homogeneity. By definition, the two fractions obtained in this way from normal serum represent what is usually understood as albumin and globulin, the characters of both of which are now, from the physico-chemical standpoint, fairly well established; but in connection with nephrotic serum these two words are used here only in their restricted original sense, namely, to designate, respectively, that part of serum (or urine) protein which precipitates at complete saturation, and that part which precipitates at half saturation, with ammonium sulfate.

When normal serum is used, the separation of the two fractions in this way is quite sharp, the filtration can be carried out immediately, and the filtrate containing the albumin fraction remains clear indefinitely. The procedure was found to be less satisfactory when dealing with nephrotic serum, though filtration could be performed more rapidly than with Howe's method (9); the filtrate obtained was usually quite transparent after an hour or two, but would not remain so more than 12 or 24 hours.

The protein solutions obtained after dialysis sometimes contained a very slight precipitate which could be filtered off easily; in case of normal albumin and urine proteins, the filtrates were water-clear; with pathological sera and normal globulin, a slight milkiness usually persisted, even after treatment with ether. Treatment with ether did not appreciably affect the osmotic pressures measured.

The nitrogen content was determined by Van Slyke's gasometric Kjeldahl method (12). For the protein:nitrogen ratio, the factors 6.41 for albumin and 6.61 for globulin, obtained by Adair and Robinson (3) for horse serum, were provisionally used.

The nephrotic subjects from whom the serum was obtained were as follows:

G. B., female, 24 years, typical nephrotic syndrome of 1 year's duration. About 35 liters of edema in November, reduced to 15 liters 2 months later. Proteinuria: 30 gm. per day. Subnormal urea clearance.

S. G., male, 9 years, typical nephrotic syndrome of 1 year's duration. Considerable ascites and edema. Proteinuria: 6 gm. per day. Normal urea clearance.

P. F., male, 3 years, and W. H., female, 33 years; both cases of nephrotic syndrome with low urea clearances.

Results of Serum Protein Studies

Table I gives the molecular weights calculated from the osmotic pressures of normal human albumin and globulin. For albumin, the figure of 72,000 may probably be taken as a reasonable average, and it appears that the mode of precipitation had no effect on the results. For globulin, the figures given for Jan. 11 should probably be chosen as the most reliable; each one of them is the average of four determinations (in each case two osmometers were used and the determinations repeated on the same sample of serum). The most trustworthy osmotic pressure measurements obtained from

animal serum yield molecular weights of about 72,000 for albumin and 170,000 or 175,000 for globulin (13); it appears that the values

TABLE I

Molecular Weights of Normal Human Albumin and Globulin Calculated from Observed Colloidal Osmotic Pressures*

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		Remarks
	Concentration	Molecular weight	
	per cent		
H. F. Oct. 1	0.342	71,600	After one crystallization
J. B. Nov. 28	0.215	71,800	Direct dialysis of albumin filtrate after globulin precipitation
	0.430	72,000	
	0.645	69,600	
	0.320	71,400	Albumin precipitated from filtrate before dialysis
	0.374	71,600	
	Globulin		
Dec. 31	2.08	162,000	One precipitation. Shaken with ether
	1.04	164,000	
	0.502	157,000	
Jan. 11	1.52	165,000	Two precipitations. Not shaken with ether
	0.760	163,000	
Dec. 9	0.615	183,000	From oxalated plasma

* Calculated by the formula

$$\text{Mol. wt.} = \frac{p_s}{p} \times c \times 10^5$$

p_s = pressure of a 0.1 mM solution at the T° of the experiment.
p = pressure of the unknown protein solution.
c = concentration of the protein solution in grams per cent.
(*Cf.* preceding publication (5) Tables I and II.)

for man are not appreciably different. Ether extraction of lipids had no effect on the results. In one case plasma instead of serum

was used, and the mean molecular weight found for the globulins was significantly higher. If one assumes that fibrinogen represents one-tenth of the total globulins, a rough calculation indicates that its molecular weight would have to be about twice that of the other globulins to cause the observed difference between plasma and serum; the point is left open for further investigation.

Table II gives the osmotic pressures developed by diluted serum both in normal and in nephrotic cases, and the theoretical pressures calculated with the help of equation 1 by taking 28.8 cm. of toluene as the pressure of a millimolar solution at 21°, assuming all the sera investigated to be a mixture of albumin with a molecular weight of 72,000 and of globulin with a molecular weight of 164,000, and taking for the concentrations the values indicated in the second column. Various diluting fluids were used, and the uniformity of the results shows that neither the salt content nor the pH of the fluid had any significant effect on the pressures developed. It appears that, whereas in the normal cases observed osmotic pressures agreed closely with those calculated from equation 1 with the molecular weights found for normal proteins, such agreement was not found for the nephrotic sera. The mean deviation of $+4$ per cent found with normal serum may be easily accounted for by the possibility that the albumin concentration is actually a little greater than the values obtained by Howe's method (9). The mean deviation of -35 per cent in the nephrotic cases indicates the presence of proteins with abnormally high molecular weights.

To ascertain whether the nephrotic proteins were in fact of abnormally high molecular weight, the osmotic pressures of albumin and globulin isolated from nephrotic sera were measured. The results, expressed as mean molecular weights, are given in Table III. The weights found for albumin are about 50 per cent higher, for globulin 100 per cent higher or more, than in normal cases. Above 2 per cent concentrations, the deviation from the van't Hoff law already becomes apparent, the molecular weights obtained being appreciably smaller than in more diluted solutions.

If we take the case of G. B., Jan. 10, (Table II) and substitute in equation 1 the values obtained on that day for albumin and globulin concentrations and those of Dec. 31 for the molecular weights (Table

TABLE II

Osmotic Pressure of Highly Diluted Human Serum
Temperature = 21°C.

Subject	Original concentration	Diluting fluid	Dilution	Pressure observed mm. toluene	Pressure calculated mm. toluene	Deviation per cent
Normal J. B. Nov. 12	*per cent* A = 5.31 G = 2.19	0.15 M NaCl	1:11 1:22 1:22	24.2 12.3 12.0	22.8 11.4 11.4	+6 +8 +5
		M/15 Sørensen's phosphate; pH = 7.65*	1:11 1:11	23.0 24.3	22.8 22.8	+1 +7
.		M/15 Sørensen's phosphate; pH = 5.88*	1:11 1:11	23.5 23.5	22.8 22.8	+3 +3
		0.1 M Na acetate 0.1 M acetic acid; pH = 4.64*	1:16 1:16	15.8 16.0	15.7 15.7	+1 +2
Nov. 23	A = 5.13 G = 2.77	0.12 N NaCl 0.03 N NaHCO₃ + CO₂†	1:11 1:22	24.3 11.7	23.1 11.5	+5 +2
Nephrotic G. B. Nov. 16	A = 1.37 G = 1.89	0.15 M NaCl	1:11 1:11	5.2 5.1	8.0 8.0	−35 −36
		M/15 Sørensen's phosphate; pH = 7.7‡	1:11 1:11	5.3 5.3	8.0 8.0	−34 −34
		0.1 M Na acetate 0.1 M acetic acid	1:11 1:11	4.9 5.1	8.0 8.0	−39 −36
Nov. 22	A = 1.61 G = 2.09	0.12 N NaCl 0.03 N NaHCO₃ + CO₂†	1:11 1:4	5.3 17.2	9.2 25.2	−42 −32
Jan. 10	A = 1.57 G = 2.15	0.15 M NaCl	1:4 1:8 1.8 1.8	16.5 8.5 8.4 8.2	25.2 12.6 12.6 12.6	−35 −33 −33 −35
S. G. Nov. 30	A = 0.82 G = 2.98	0.15 M NaCl	2:7 2:7	15.0 15.5	24.4 24.4	−39 −36

* Determined with the glass electrode after dilution.
† This mixture was made by bubbling expiratory air through the solution.
‡ Calculated value before dilution.

III), the theoretical pressure for a serum diluted four times would be
(taking 28.8 cm. of toluene as the pressure of a mM solution at 21°)

$$\left(\frac{15,700}{102,000} + \frac{21,500}{240,000}\right) \times 28.8 \times 1/4 = 17.6 \text{ mm. of toluene,}$$

instead of the 16.5 mm. observed.   For a dilution of $\frac{1}{8}$ the calculated
values would be 8.8 mm. instead of the 8.2 to 8.5 observed.

TABLE III

*Molecular Weights of Serum Albumin and Globulin in Nephrotic Subjects
Calculated from Colloidal Osmotic Pressures*

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin.

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	per cent		*per cent*	
G. B. Nov. 27	0.166	105,000		
	0.196	106,000		
Dec. 31	0.442	104,000	2.13	217,000
	0.628*	104,000	1.07	240,000
	0.314*	99,000		
S. G. Nov. 29	0.196	122,000		
Dec. 16			1.34	298,000
Dec. 31			2.17	314,000
			1.09	353,000
			0.504	346,000

* In these two samples the lipids were extracted with ether after dialysis,
after which the sample was redialyzed for 2 hours.

More accurate calculations are probably not warranted, since the
proteins were precipitated with ammonium sulfate for the molecular
weight determinations, and with sodium sulfate for the determinations
of concentration.   The two salts have been found to precipitate
approximately the same amounts of globulin in normal serum, but
whether the same holds for nephrotic serum has not been investigated.

2. *Nature of the Proteins in the Urine of Nephrotic Patients*

The earlier literature about proteinuria has been reviewed by Hiller *et al.* (8), who have found that in nephrosis the albumin:globulin ratio is usually above 10, whereas it is usually lower in glomerulonephritis. It has been thus far generally assumed that the albumin and globulin of urine in Bright's disease were identical with the albumin and globulin of normal serum. McFarlane (11), however,

TABLE IV

Molecular Weights of Urine Albumin and Globulin in Nephrotic Subjects Calculated from Colloidal Osmotic Pressures

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	per cent		*per cent*	
S. G. Dec. 1	0.560	61,700		
	0.187	62,000		
G. B. Dec. 5	0.439	61,600	1.24	114,000
			0.620	120,000
Jan. 23			0.712	107,000
P. F. Dec. 5	0.555	66,700		
W. H. Dec. 12	0.403	57,200	1.13	120,000

has observed that the urine albumin of patients with Bright's disease is less homogeneous in the ultracentrifuge than is normal albumin.

Methods

For the preparation of albumin and globulin fractions urine was treated like serum, except that treatment with ether was omitted. The albumin-containing filtrate, after globulin precipitation, was always perfectly clear, as that of normal serum. Osmotic pressures were measured as indicated above.

Results with Urine Proteins

Table IV gives the results. The molecular weights calculated from the osmotic pressures of the urine albumin and globulin are definitely below the weights obtained for the albumin and globulin of normal serum; the difference is more marked for globulin than for albumin.

DISCUSSION

It goes without saying that the molecular weights given for albumin and globulin fractions in the tables should be assumed to indicate only the average sizes of the molecules in each fraction. The globulin fraction, from its salting-out curves, electrophoretic behavior (14) etc., is in general believed to include more than one distinct protein, and the albumin fraction of nephrotic serum appears also to be heterogeneous. Of the fractions here studied, the albumin of normal serum is the only one that, from its physicochemical behavior and its crystallizability, may perhaps be homogeneous. We have not been able to crystallize the albumin of nephrotic patients, either from serum or from urine.

McFarlane (11) has studied the sedimentation rates in the ultracentrifuge of the serum and urine proteins of five cases of proteinuria. One of these was apparently a case of nephrosis, another a case of nephritis. In all cases the serum showed the presence of polydisperse albumin. Our conclusions are therefore in accord with his.

The data of Table II indicate that the pressure of highly diluted serum can be expressed as the sum of the partial pressures of albumin and globulin, if correct figures for the specific pressure, or molecular weight, of each fraction are used in the calculation. This appears to be true not only for normal serum, but also for nephrotic serum, in which the molecular weights of both albumin and globulin, as separated with ammonium sulfate, were found to be from 50 to 100 per cent higher than in normal serum.

Since we are still in ignorance as to the laws which govern deviation from the van't Hoff law, it is probably not warranted to compare the results presented here with those obtained from osmotic pressure measurements on higher concentrations, and without attempt to estimate specific pressures at infinite dilution. This statement applies to the formulas, calculated by Govaerts and his followers (7, 16, 18),

relating the osmotic pressures of undiluted serum to its albumin and globulin concentration, and also to Widdowson's results (17).

The results of Table IV show that the urine proteins of the nephrotic patients differ from serum proteins of the same patients even more markedly than from the serum proteins of normal subjects. Roughly, the mean molecular weight of urine albumin is one-half that of serum albumin, that of globulin one-third the molecular weight of serum globulin, from the same patient.

These results support the idea that in proteinuria the kidney is more permeable to the proteins of smaller molecular size. It has long been known that albumin passes more abundantly than globulin into the urine (8). Our results indicate that, from the heterogeneous albumin fraction of nephrotic serum, the subfractions of lower molecular size pass more abundantly into the urine; and similarly for the subfractions of the globulins.

SUMMARY

In serum of patients with nephrosis both albumin and globulin showed by osmotic pressure nearly double the molecular weights of normal albumin and globulin.

In the urines of such patients, on the other hand, both proteins showed molecular weights lower even than in normal serum.

The colloidal osmotic pressures were measured by the author's method at such dilutions that the van't Hoff law relating pressures to molecular concentrations could be directly applied. For the albumin and globulin of normal serum the molecular weights found were 72,000 and 164,000 respectively, in agreement with the weights obtained by other methods.

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EXPERIMENTAL HYPERTENSION

The Effects of Unilateral Renal Ischemia Combined with Intestinal Ischemia on the Arterial Blood Pressure*

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PLATE 43

Longcope and McClintock (1) determined the effects of constriction of the celiac axis and superior mesenteric artery of dogs by means of aluminum bands and found that neither cardiac hypertrophy nor hypertension followed the narrowing of these vessels. Blalock and Levy (2), by multiple stage operations, produced complete occlusion of the celiac axis, superior and inferior mesenteric arteries in survival experiments on dogs and reported that these procedures do not result in arterial hypertension. However, the blood pressure did not return entirely to the control level in approximately one half of the animals which survived following complete occlusion of the three main intestinal vessels, remaining elevated about 15 to 20 mm. Hg. This was considered to be of no significance but in view of later observations, it may be of importance.

The present experiments consist of a study of the effects on the blood pressure of unilateral renal ischemia in animals in most of which the three main intestinal vessels had been occluded previously.

Methods and Results

Dogs were used in all experiments. The blood pressure was determined by puncturing the femoral artery with a needle that was con-

nected to a mercury manometer. The value obtained is approximately the mean blood pressure. The celiac axis, superior and inferior mesenteric arteries were occluded completely in multiple stage operations by the use of Goldblatt clamps.[1] In four experiments, unilateral renal ischemia was not produced until after the occlusion of these intestinal vessels had been rendered complete. In one experiment, due to an oversight, unilateral renal ischemia was caused before the inferior mesenteric artery was occluded. In the sixth experiment, intestinal and unilateral renal ischemia were induced simultaneously.

Since the experiments were few in number and since the procedures and results varied somewhat in the different studies, the protocol of each experiment is given in a moderate amount of detail. The two figures for blood pressure are the high and low values associated with the act of respiration.

Dog 1. Aug. 5, 1937, control blood pressure (needle puncture) 125–130. Aug. 5, incision left flank, Goldblatt clamps to celiac axis, one of $2\frac{1}{8}$ turns, to superior mesenteric artery $\frac{3}{4}$ of $2\frac{3}{4}$ turns. Slight temporary rise in blood pressure. Aug. 24, blood pressure 125–130. Aug. 25, $\frac{3}{4}$ additional turn of each clamp. Sept. 20, blood pressure 110–125. Abdominal incision, Goldblatt clamp to inferior mesenteric artery $2\frac{1}{4}$ of $3\frac{1}{4}$ turns. Oct. 23, blood pressure 142–152, incision left flank. Clamp on superior mesenteric artery had cut through artery, clamp came out when grasped. Clamp on celiac axis completely tightened. Nov. 4, blood pressure 160–166, abdominal incision, arterial pulsations could not be felt in intestinal vessels. Clamp on inferior mesenteric artery completely closed. The blood pressure the succeeding 2 months averaged approximately 150 mm. Hg. Jan. 14, 1938, incision left flank, Goldblatt clamp to left renal artery two of $3\frac{1}{2}$ turns. Jan. 19, blood pressure 210–215. The mean blood pressure which was determined every 2 or 3 days has remained at approximately that level. Aug. 3, blood pressure 205–215, incision right flank, the right kidney and renal vessels seemed entirely normal. Kidney possibly somewhat hypertrophied. Pulsations could be felt in arteries in mesentery of intestine but they were thought to be diminished. Jan. 31, 1939, blood pressure 215–225. Feb. 18, blood pressure 200–210. This dog lost weight during the process of occlusion of the vessels to the intestinal tract but has more than regained it since that time. Despite the fact that a clamp was applied to the renal artery of only one kidney, there has been a persistent elevation in blood pressure for almost 14 months. The blood pressure readings are recorded in Text-fig. 1.

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[1] Furnished through the courtesy of Dr. Harry Goldblatt, Cleveland, Ohio.

Dog 2. Feb. 2 to Feb. 10, 1938, control blood pressure (needle puncture) varied from 105 to 130 mm. Hg. Weight 8.5 kg. Feb. 10, incision left flank, Goldblatt clamps to celiac axis, $\frac{3}{4}$ of $3\frac{1}{4}$ turns, to superior mesenteric artery, $\frac{2}{3}$ of $3\frac{1}{2}$ turns, to left renal artery $1\frac{5}{6}$ of $3\frac{1}{4}$ turns. A moderate rise in the blood pressure followed. Mar. 5, blood pressure 160–170. Incision left flank. Clamps on celiac axis and superior mesenteric artery each tightened $\frac{3}{4}$ turn, clamp on renal artery tightened $\frac{1}{4}$ turn. Mar. 16, blood pressure 150–165, inferior mesenteric artery doubly ligated and divided. Apr. 15, blood pressure 158–166. Apr. 19, blood pressure 130–138. Weight 8.2 kg. Apr. 21, clamps on celiac axis and superior mesenteric artery completely closed. Clamp on renal artery tightened $\frac{1}{4}$ turn. Apr. 30, blood pressure 175–185. The mean blood pressure continued to range
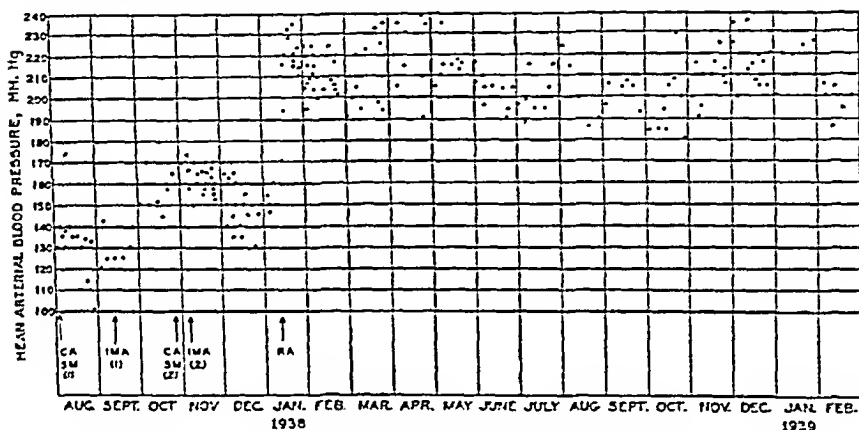
Text-Fig. 1. Dog 1. • = mean arterial blood pressure. CA, SM (1), partial constriction of celiac axis and superior mesenteric artery. IMA (1), partial constriction of inferior mesenteric artery. CA, SM (2), celiac axis and superior mesenteric artery completely occluded. IMA (2), inferior mesenteric artery completely occluded. RA, partial constriction of left renal artery.

between 170 and 240 mm. Hg, the usual level being about 195 mm. Hg. Nov. 16, blood pressure 182–188, incision left lower chest, supradiaphragmatic splanchnicectomy on left. Nov. 23, blood pressure 195–205. Dec. 2, blood pressure 190–200. Dec. 3, supradiaphragmatic splanchnicectomy on right. Dec. 5, blood pressure 205–215. Feb. 9, 1939, blood pressure 190–198. Ether, incision right flank. Right kidney and artery normal, incision closed. Feb. 11, blood pressure 190–200. Feb. 20, blood pressure 190–200. Weight 7.4 kg. It is now more than 10 months since the Goldblatt clamp on the renal artery was tightened the last time and the blood pressure has remained elevated. The blood pressure readings are given in Text-fig. 2.

Dog. 3. Feb. 25, 1938, weight 8.0 kg. Control blood pressure (needle puncture) 124–130. Incision left flank, Goldblatt clamps to celiac axis, 1¼ of 2¾ turns, to superior mesenteric artery ¾ of 3½ turns. Slight temporary rise in blood pressure. Mar. 18, blood pressure 114–122. Incision left flank, ½ turn to clamp on celiac axis and one turn to that on superior mesenteric artery. Temporary rise in blood pressure. Apr. 8, inferior mesenteric artery doubly ligated and divided. May 17, blood pressure 145–155. Incision left flank, the occlusion of the Goldblatt clamps on the celiac axis and superior mesenteric artery was made complete. Slight rise in pressure. June 14, blood pressure 140–145. Incision right flank, Goldblatt clamp to right renal artery 1⅝ of three turns. June 20, blood pressure 190–196.
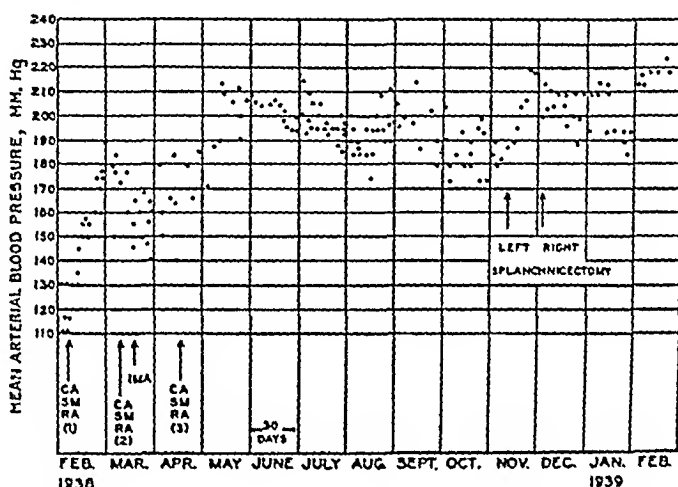
Text-Fig. 2. Dog 2. • = mean arterial blood pressure. CA, SM, RA (1), partial constriction of celiac axis, superior mesenteric artery and left renal artery. CA, SM, RA (2), constriction of these three vessels increased. IMA, ligation and division of inferior mesenteric artery. CA, SM, and RA (3), celiac axis and superior mesenteric artery completely occluded, constriction of left renal artery increased. Right and left splanchnicectomy, supradiaphragmatic approach.

June 21, blood pressure 220–230. It is now more than 8 months since this single application of the Goldblatt clamp to the renal artery of one of the two kidneys, the blood pressure has remained elevated, the mean pressure ranging from 170 to 215 mm. Hg. Weight of animal 10.0 kg. The blood pressure readings are given in Text-fig. 3.

Dog. 4. Nov. 5, 1937, mean blood pressure 115–120. Incision left flank. Goldblatt clamp to celiac axis 1¼ of 3¼ turns, to superior mesenteric artery ½ of 3¼ turns. Little if any alteration in blood pressure followed. Nov. 26, blood pressure 108–116, incision left flank, each clamp tightened ¾ turn. Dec. 16, blood pressure 122–130, clamp on inferior mesenteric artery 2¼ of 3¼ turns. Slight

rise in pressure followed.   Jan. 3, 1938, blood pressure 138–144, incision left flank, clamps on celiac axis and superior mesenteric artery completely tightened.   Jan. 11, blood pressure 142–146, lower abdominal incision, clamp on inferior mesenteric artery completely closed.   No pulsations could be felt in mesenteric vessels.   Jan. 24, blood pressure 128–134, incision right flank, Goldblatt clamp to right renal artery, 1¾ of 3½ turns.   Moderate rise in blood pressure.   Feb. 10, blood pressure 175–180.   Incision right flank, ½ turn of clamp on renal artery.   Feb. 14, blood pressure 204–210.   Mar. 16, blood pressure 170–175.   Incision right flank, ¼ turn of clamp on renal artery.   Mar. 26, blood pressure 175–180.   The mean blood pressure remained at approximately this level.   July 2, blood pressure 175–180.   July 7, blood pressure 155–160.   Dog has developed discharging sinus
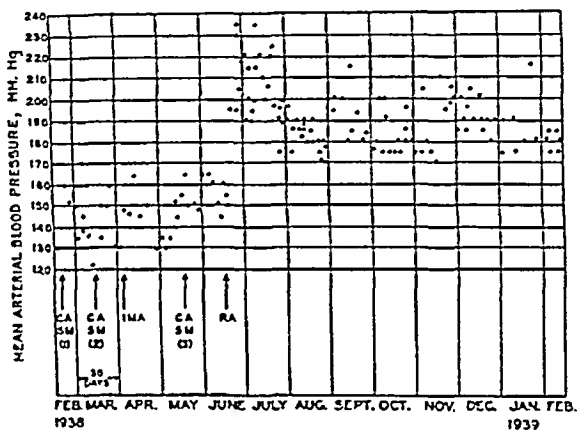
TEXT-FIG. 3. Dog 3.   • = mean arterial blood pressure.   CA, SM (1), partial constriction of celiac axis and superior mesenteric artery.   CA, SM (2) increase in constriction of same two vessels.   IMA, ligation and division of inferior mesenteric artery.   CA, SM (3), complete occlusion of celiac axis and superior mesenteric artery.   RA, partial occlusion of right renal artery.

right flank.   Incision right flank.   Enormous right kidney with marked pyonephrosis.   Autopsy showed complete occlusion of right renal artery, celiac axis, superior and inferior mesenteric arteries.   Left kidney and left renal artery appeared normal.   The blood pressure remained definitely elevated for more than 5 months following constriction of the artery to one of the two kidneys.   It began to decline when a severe pyonephrosis developed.

Dog 5.   Weight 12.4 kg.   Control blood pressure (needle puncture) 120–130 mm. Hg.   Feb. 25, 1938, incision left flank, Goldblatt clamps to celiac axis 2¼ of four turns, to superior mesenteric artery ½ of 3½ turns.   Feb. 28, blood pressure 156–162.   Slow return of blood pressure to normal.   Mar. 18, blood pres-

sure 110–120. Incision left flank, ¾ turn to clamp on celiac axis and 1½ to clamp on superior mesenteric artery. Slight rise in blood pressure. Apr. 9, inferior mesenteric artery doubly ligated and divided. Slight rise in blood pressure. May 17, blood pressure 135–140, clamps on celiac axis and superior mesenteric artery completely closed. Very little change in blood pressure. June 9, blood pressure 130–135. Weight 11.9 kg. June 14, blood pressure 145–150. Incision right flank, Goldblatt clamp to right renal artery, two of three turns (pulsation obliterated at 2½ turns). June 17, blood pressure 160–165. July 2, blood pressure 184–190, weight 11.8 kg. July 11, blood pressure 165–170. Incision right flank, kidney smaller than normal. Right main renal artery found to be completely occluded. Pulsations could be felt in ureteral vessels. July 15, blood pressure 165–170. The blood pressure varied during the succeeding 4 months between 140 and 180. Nov. 7, blood pressure 160–165, incision left flank, Goldblatt clamp to left renal artery, 4/5 of 2⅘ turns. Nov. 12, blood pressure 190–195. Nov. 16, blood pressure 210–215. Dog looked sick. Incision left flank reopened, blood clot around clamp, clamp loosened. Nov. 21, blood pressure 230–236, N.P.N. 28. Marked elevation in blood pressure continued. Dec. 5, blood pressure 270–275. Dec. 12, weight 12.4 kg. Blood pressure 250–260. Given large dosages of phlorizin for 4 days at suggestion of Dr. Morton F. Mason in order to study its effect on the blood pressure. The pressure remained markedly elevated. Dec. 19, blood pressure 250–260. Dec. 24, blood pressure 235–245, animal appeared to be in good condition, good appetite. No abnormality noted except that eyes had been rather prominent for several weeks. Dec. 25, animal died, apparently rather suddenly. Autopsy revealed the following findings. Bloody fluid in both pleural cavities. The pericardium was filled with blood, most of which was clotted. The clot weighed 90 gm. There was a hemorrhagic area overlying the intrapericardial portion of the aorta with hemorrhage into a cavity which appeared to be between the adventitia and media of the wall of the aorta. There was a peculiar small defect overlying the left descending coronary artery. On one occasion it was thought that a small blood clot was squeezed from it but this could not be repeated. The kidneys were essentially normal in size, the left slightly smaller than the right. The left main renal artery was occluded. The lumen of the main right renal artery was occluded but there was a smaller artery which was patent. Hemorrhagic area just beneath the capsule of the left kidney. The celiac axis, superior and inferior mesenteric arteries were found to be completely occluded. The intestinal tract appeared to be essentially normal.

Dr. James Dawson of the Department of Pathology studied the microscopic sections and gave us the following report:

Examination of sections from the aorta shows that the wall of this vessel has been split. This is shown in Fig. 1. This splitting of the wall is seen in the outer third of the media. In general the coats of this vessel appear normal except for the blood filled space. There are, however, focal areas in which there is acute medio-necrosis associated with a polymorphonuclear leucocytic infiltration. This necrosis occurs in the outer portion of the media chiefly. Verhoeff elastic tissue

stains show little alteration in the elastic fibrils; there is questionable fraying but nothing more. The vasa vasorum in general have narrowed lumina; the degree of narrowing varies considerably but some are almost obliterated. This is shown in Fig. 2. This narrowing is due chiefly to intimal thickening. The split in the vessel wall generally is longitudinal for the most part but in some instances one sees clefts which run at right angle to the long axis of the vessel. These have extended through to the adventitia and within them as well as in the peri-adventitial stroma there is massive, fresh hemorrhage.

There is narrowing of the lumina of the smaller arteries and arterioles in the retina due to increase in thickness of media and intima. In the brain a few minute areas of softening are seen but there is little or no change in the vessel walls. The smaller branches of the coronary arteries show no definite change.

It is impossible to be certain of the sequence of events which transpired but it seems likely that obliteration of the lumina of the vasa vasorum, due to intimal thickening, led to necrosis of the media with the weakening of the wall; with the coexistent hypertension, rupture occurred and blood was forced into the vessel wall where it dissected along the media, finally escaping into the pericardium and leading to the death of the animal.

Dog 6. Male, weight 10.2 kg. Control blood pressure (needle puncture) 130–135. Feb. 22, 1938, incision left flank, Goldblatt clamp to celiac axis, ¾ of 3 turns, to superior mesenteric artery, ¾ of 3 turns. Feb. 25, blood pressure 140–146. Mar. 14, blood pressure 120–125, incision left flank, each Goldblatt clamp tightened ¾ turn. Mar. 25, weight 7.75 kg., blood pressure 110–115. Blood pressure remained at approximately this level. May 18, constriction of celiac axis and superior mesenteric artery was made complete. June 14, blood pressure 124–130, incision left flank, Goldblatt clamp to left renal artery 1⅜ of 3¼ turns (occluded pulsation at 2¼ turns). June 16, blood pressure 150–155. June 20, weight 9.3 kg., blood pressure 140–145. June 25, clamp on left renal artery tightened ¼ turn. July 2, weight 8.81 kg. Blood pressure 130–135. Abdominal incision. Inferior mesenteric artery which was approximately twice the normal size was doubly ligated and divided. Very little alteration in the blood pressure. July 16, weight 9.27 kg., blood pressure 135–140. Incision left flank, kidney approximately one-fourth normal size. The clamp had cut through the artery. Its removal was followed by some bleeding, renal artery and vein ligation necessary in control of hemorrhage. No pulsations felt in ureteral vessels. Blood pressure slightly elevated for several days, return to approximately 135 mm. Hg where it remained. Oct. 20, weight 10.3, blood pressure 130–135. Nov. 7, incision right flank, Goldblatt clamp to right renal artery, one of 2¼ turns. Nov. 12, blood pressure 120–125. Dec. 12, blood pressure 140–145. Dec. 22, blood pressure 140–145, incision right flank, constriction of right renal artery increased by ½ turn of clamp. Dec. 29, blood pressure 175–180. Jan. 9, 1939, blood pressure 215–225. Animal appeared ill, N.P.N. 71. Jan. 14, blood pressure 200–206. Animal appears well. Weight 10.4 kg. Feb. 4, blood pressure 205–215. Feb. 13, blood

pressure 220–225. Feb. 20, blood pressure 210–216. There was very little elevation in this animal until the second renal artery was constricted. It may be of significance that the Goldblatt clamp completely occluded the first renal artery.

DISCUSSION

It would appear to be more than a coincidence that a prolonged elevation in the blood pressure was observed in four of the six animals in which occlusion of the main arteries to the intestinal tract was followed by constriction of the renal artery of one of the two kidneys. As has been stated the pressure has remained definitely elevated for 14, 10, and 8 months respectively in three of the animals. The pressure declined in the fourth animal coincident with the development of pyonephrosis which was 5 months after the production of unilateral renal ischemia. The rise in pressure in the fifth animal was not so great and was not sustained as long. This may have been due in part to an accessory renal artery of moderate size which was found at the time of autopsy. It is interesting that this animal developed the most marked hypertension that we have observed following the induction of bilateral renal ischemia. Death was due to rupture of the intrapericardial portion of the aorta with resulting cardiac tamponade. There was very little elevation in the pressure in the sixth animal. It was found on exploration that the Goldblatt clamp had occluded completely the renal artery. In two of the four animals in which unilateral renal ischemia resulted in the most marked and sustained elevation in the blood pressure, the pressure was approximately 20 mm. higher before renal constriction than it had been prior to the production of intestinal ischemia. This may be an important factor in determining the response to renal ischemia. In general, the animals which exhibited the most marked rise in pressure associated with intestinal ischemia showed an even more marked elevation when unilateral renal ischemia was caused. This relationship could not be tested in one of the animals since intestinal and renal ischemia were induced simultaneously. A greater number of experiments are desirable and should be performed. The reasons for the relatively few experiments reported here are the high mortality rate associated with progressive intestinal ischemia, the technical difficulties associated with repeated exposure of the clamps, and the long time that it is necessary to observe these animals.

That the rise in blood pressure associated with unilateral renal ischemia was not due to the absence of a second kidney was shown by an exploratory operation in which the kidney and renal artery were palpated. Constriction of the intestinal vessels was not observed to reduce the calibre of the aorta with a resulting effect on the renal circulation. The operations for the purpose of inducing intestinal ischemia were all performed through an incision in the left flank. In the procedure of producing unilateral renal ischemia, the right and left sides were chosen an equal number of times. That the rise in blood pressure was not due to scarring of the renal pedicle associated with the operations on the intestinal vessels was shown by the absence of adhesions in the neighborhood of the right renal artery. Furthermore, we were unable to produce hypertension in previous experiments by injecting sclerosing solutions (sodium morrhuate) into and around the walls of the two renal arteries.

As stated, the prolonged elevation in blood pressure in four of these animals with unilateral renal ischemia would seem to be more than a coincidence. In a number of controls in which intestinal ischemia has not been present, we have not observed as lengthy and as marked elevations in pressure associated with unilateral renal ischemia. The usual finding is an elevation in pressure for 2 or 3 weeks followed by a return to the control level.

The following has been the experience of others. Goldblatt (3) stated, "It was shown in the original communication that hypertension of some degree follows the constriction of the main renal artery of only one kidney but that after a variable period the blood pressure tends to return to the original level. In some dogs the blood pressure remains elevated for a considerable period following unilateral renal ischemia. In one dog, the mean blood pressure remained at a higher level than normal for about nine months following the constriction of the main renal artery of only one kidney. During this time there was no impairment of renal function. After the removal of the kidney the blood pressure promptly fell to the normal level." The control pressure in this animal was approximately 135 mm. Hg and the pressure was approximately 180 mm. Hg 9 months following the production of unilateral renal ischemia. Goldblatt (4) stated subsequently, "In these and other studies, some of which have been reported, it has been found that when one renal artery is constricted adequately, the blood pressure rises in most animals and remains elevated for a variable period which lasts from weeks to months, but eventually returns to a lower, or even the original, level. In order to make the hypertension persist for years, it is necessary either to clamp the main

artery of the other kidney or to remove the normal kidney." In a personal communication (1938), Goldblatt states that the most common experience following unilateral constriction is an elevation in pressure which lasts from 2 to 6 weeks. Wood and Cash (5) have observed one dog in which the blood pressure remained elevated for 2 years following the production of unilateral renal ischemia. The animal was a very "nervous" male which weighed 27 pounds at the time that the right renal artery was constricted. Using their method for determining the systolic and diastolic pressures, the control figures were 148 and 54 mm. Hg. 2 years subsequently, the corresponding figures were approximately 200 and 83 mm. Hg. There had been a gain in weight of 10.5 pounds during this period. This may be of some significance. One of our animals has shown a considerable gain in weight. Elaut (6) produced hypertension by the Goldblatt method. He stated, "The hypertension is least pronounced and hardly exceeds 190 mm. Hg when the compression is limited to only one renal artery, the other remaining intact. It rapidly exceeds 200 mm. Hg if one compresses equally the two renal arteries." Verney and Vogt (7) report that the blood pressure was still elevated in one dog 104 days after unilateral renal ischemia had been induced. Fasciolo, Houssay, and Taquini (8) state, "Rise in blood pressure is obtained when ischemia of either or both kidneys is produced. If only one kidney is involved the blood pressure usually increases less and more slowly than if the two are involved; moreover, after a time it has a tendency to fall gradually to the normal level."

Additional confirmation of the rarity of a sustained elevation in the blood pressure following unilateral renal ischemia was obtained in the following additional observations. The first of these consisted of constricting partially one renal artery in each of five animals in which the two carotid and the two vertebral arteries had been ligated and divided previously. The latter procedure resulted in a moderate temporary elevation in the blood pressure. The production of unilateral renal ischemia was followed in four of the five animals by an elevation in blood pressure of about 35 mm. Hg which lasted for approximately 3 weeks. A rise in blood pressure of about 35 mm. Hg which persisted for 4.5 months, at which time the experiment was terminated, was noted in the fifth animal. The second observations consisted of producing unilateral renal ischemia in seven dogs after the pressure had returned to normal following ligation and division of the two common iliacs and the two subclavian arteries. There was no definite elevation in the pressure in one of these. There was an elevation in pressure of approximately 35 mm. Hg in four of these and it remained elevated for periods ranging from 3 to 8 weeks. The pressure of one of the animals rose approximately 50 mm. and remained

elevated for 11 weeks, at which time it returned to normal. The pressure of the seventh animal was elevated approximately 45 mm. for 3 months. It gradually declined after this time but did not quite return to the control level.

In one animal reported by Goldblatt (9), the splenic artery and both femoral arteries were greatly constricted at different times before the clamps were applied to the renal arteries, but no rise of blood pressure occurred until after the renal arteries were constricted.

That the non-ischemic kidney may be a factor in determining the blood pressure response to unilateral renal ischemia was shown by Blalock and Levy (10). In six of eight animals in which the blood pressure had returned to normal following the production of unilateral renal ischemia, removal of the sound kidney was followed by a re-elevation of the blood pressure. These findings have been confirmed by Verney and Vogt (7) and by Fasciolo, Houssay, and Taquini (8).

Various operations on the nervous system, even total sympathectomy (11), do not prevent the development of or abolish the hypertension that is associated with renal ischemia. It is evident that the renal ischemia cannot be altered by these procedures as long as the clamps remain applied. Bilateral supradiaphragmatic splanchnicectomy was performed on one of our animals with marked hypertension associated with intestinal and unilateral renal ischemia. No alteration in the blood pressure followed these procedures.

The cause for the unusually great and well sustained elevation in the pressure in most of the animals with intestinal and unilateral renal ischemia is not apparent. Even if one may for the moment liken occlusion of the large vessels of the intestinal tract to arteriosclerosis of the great vessels, it is well known that changes in the smallest arteries or arterioles are more closely correlated with hypertension than are lesions of the large arteries. A low blood pressure may be present in an individual with advanced arteriosclerosis. Extensive arteriosclerosis of the vessels of the gastro-intestinal tract is encountered very rarely. Occlusion of the large vessels to the intestinal tract at their origins from the aorta undoubtedly results in the opening up of many small vessels. This almost certainly results in an increase in the resistance to the flow of blood. This increase in resistance is apparently not great enough on its own account to cause a marked permanent elevation in the blood pressure, the disappearance being due

very likely to the opening up of collaterals. However, the small elevation in pressure which persisted in some of the animals following the induction of intestinal ischemia may be of more importance than we have assumed. Verney and Vogt (7) have shown recently that the perfused (non-ischemic) kidney liberates a substance which causes vasoconstriction of the vessels of the intestinal tract. The present conception of the hypertension associated with experimental renal ischemia is that a pressor substance (12, 13, 8) is formed in the kidney which causes increased peripheral vasoconstriction. It is possible that the explanation for our findings lies in an increased effectiveness of this hypothetical pressor substance due to an increase in the number of small vessels on which it may exert its effects. According to Poiseuille's law, the pressure varies inversely as the fourth power of the radius of a very small vessel. An extremely small increase in the tonus of a large area of arterioles suffices to cause marked hypertension. Goldblatt (4) has described thickening of the media of the arterioles with or without hyalinization of the intima in chronic hypertension due to renal ischemia. Child (14) has observed widespread changes in the arterioles, consisting of thickening of the walls with a decrease in the size of the lumina, and says that these alterations are most marked in the cardiac and mesenteric vessels. Unilateral renal ischemia may cause such changes in some of the smaller vessels conveying blood to the intestinal tract. This, however, is speculation as the animals which have had hypertension for long periods are still alive. It is interesting that one of our animals developed such marked alterations in the wall of the ascending aorta following the application of a clamp to the second renal artery that rupture of this large vessel occurred. This is the first time that this has been observed. Another possibility is that intestinal ischemia may in some way interfere with the development of collateral circulation to the ischemic kidney. Still another possibility is that intestinal ischemia may in some way interfere with the destruction of the pressor substance, if the evidence to the effect that such is formed in the ischemic kidney is correct.

Heymans (15) has called attention to a pressoreceptive mechanism located in the splanchnic area which has to do with the regulation of blood pressure and other functions. He states, "Experiments employing several different methods have permitted us, with the collaboration of Bouckaert, Farber, Hsu and Wierzu-

chowski, to show that in the dog deprived of carotid sinus, cardio-aortic, pulmonary and venous presso-sensitive zones, as in the spinal animal, the proprioceptive regulation of vascular zone still occurs, through presso-sensitive reflexes originating mostly from the vascular territory of the celiac and mesenteric arteries and secondarily from the territory of the thoracic arteries." It is possible that intestinal ischemia increases the responsiveness of this regulating mechanism to the effects of the hypothetical renal pressor substance.

Longcope and McClintock (1) examined at autopsy 46 patients in whom either the heart showed hypertrophy or the mesenteric artery and celiac axis were narrowed by arteriosclerotic processes, pressures from aneurisms, or from new growths. Of the 21 cases in which the splanchnic vessels were narrowed, only seven were associated with cardiac hypertrophy and in all seven cases a chronic nephritis coexisted.

The experiments reported confirm this observation in that intestinal ischemia alone does not result in a marked permanent elevation in the blood pressure.

SUMMARY

Unilateral renal ischemia superimposed upon intestinal ischemia has resulted in a prolonged elevation in the arterial blood pressure in a high percentage of the animals which were studied.

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EXPLANATION OF PLATE 43

FIG. 1. Hematoxylin and eosin. Photomicrograph of longitudinal section of aorta showing the split in the media and hemorrhage into the media and adventitia. × 30.

FIG. 2. Hematoxylin and eosin. Photomicrograph showing vasa vasorum in media of aorta with narrowing of lumen due to thickening, particularly in intima. × 500.

SEROLOGICAL STUDIES OF SWINE INFLUENZA VIRUSES

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Most of the work with swine influenza virus has been carried out with strain 15, recovered originally in Iowa in 1930. Prior to 1937 this strain was, from time to time, superficially compared with swine influenza viruses obtained in different epizootic outbreaks, and no evidence to indicate immunological heterogeneity among the various strains was detected. Judgment of the identity of the viruses being compared was usually based upon their ability to produce cross-immunity in swine, though some cross-neutralization tests with sera of recovered swine or ferrets failed to detect strain differences either. Swine influenza viruses compared in this way with strain 15 or with one another and considered on the basis of the results obtained to be immunologically identical were strain 14 (Iowa, 1930), strain 17 (Iowa, 1931), strain 18 (Iowa, 1932), strain 19 (Iowa, 1933), strain 20 (Iowa, 1934), and strain 23 (Ohio, 1935).

During the early years of work with human influenza virus, investigators recovered strains from patients in different epidemics and widely separated localities. These viruses from man were assumed, largely on the basis of cross-immunity tests in ferrets, to be immunologically identical. In 1936, however, Magill and Francis (1), using virus-neutralizing serum prepared in a non-susceptible host (rabbit), obtained evidence that their Puerto Rico and Philadelphia strains differed antigenically. Later Burnet (2), Andrewes (3), and Andrewes, Smith, and Stuart-Harris (4) demonstrated serological differences among other strains of human influenza virus. Recently the question of immunologic variation among the large number of strains of human influenza virus now available for study has been thoroughly investigated by Magill and Francis (5, 6) in this country and by Smith and Andrewes (7) in England. The conclusions reached in both investigations were that there is great immunological diversity among strains of human influenza virus and that the virus is antigenically complex. Smith and Andrewes believed that their experiments indicated the existence of at least 4 major antigenic com-

ponents among the 28 strains of virus they studied. They classified the strains, on the basis of their content of the 4 major antigens, into 3 main categories, namely, highly specific strains, relatively non-specific strains, and intermediate strains. Magill and Francis classified their 24 strains into 6 groups as determined by serological similarities or differences and pointed out that the strains which most closely resembled one another were, in general, those from the same epidemic of influenza. Serologically different strains were, however, also recovered from the same epidemic.

These observations concerning serological diversity among strains of the human influenza virus raised the question of whether or not similar variations existed among strains of the swine influenza virus recovered in different epizootics. The experiments reported in this paper were conducted in an attempt to answer the question.

Materials and Methods

Strains of Virus.—The human influenza viruses employed in the present experiments were strains WS, PR8, and Oakham, recovered respectively from cases of epidemic influenza in 1933, 1934, and 1937.[1] The swine influenza viruses used were strain 15 (Iowa, 1930), strain 20 (Iowa, 1934), strain 23 (Ohio, 1935), strain 24 (Nebraska, 1936), strain 28 (Iowa, 1936), strain BC (New Jersey, 1936), and strain 29 (Iowa, 1937).

All strains of virus studied serologically were well adapted to white mice before use in the present experiments and were of such pathogenicity that the supernatant of a 1 per cent infected lung suspension killed all mice inoculated intranasally in less than 5 days. Virus suspensions both for use in neutralization experiments and for the immunization of rabbits were prepared from glycerolated infected mouse lungs.

Sera.—The swine sera were obtained by tail or heart bleeding 11 to 13 days after infection with swine passage swine or human influenza virus mixed with a small amount of a culture of the bacterium *Hemophilus influenzae suis* (8). The swine furnishing the sera were thus in early convalescence.

The rabbit sera were obtained by marginal ear vein bleeding on the 10th and 13th days after intraperitoneal injection with 7 cc. of a 5 per cent suspension of mouse lung infected with either swine or human influenza virus. The 10th and

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[1] I am indebted to Dr. C. H. Andrewes for the WS strain, Dr. Thomas Francis, Jr., for the PR8 strain, and Dr. C. H. Stuart-Harris for the Oakham strain.

13th day bleedings from each rabbit were pooled for use in the neutralization tests. This method of immunization differs somewhat from that employed by Magill and Francis (5) in that they bled their rabbits on the 8th day, and they graded their immunizing dose to correspond roughly with the titer of the virus strain being used.

All sera, both from swine and rabbits, were filtered through Seitz pads prior to storage in the refrigerator until used.

Neutralization Tests.—The neutralization tests were conducted in white mice by the technique regularly used in this laboratory (9).

The supernatant of a 2 per cent suspension of glycerolated infected mouse lung was employed as virus, and this was mixed in equal parts with the undiluted sera to be tested. The mixtures were stored for 2 hours in the refrigerator prior to their administration to white mice. 3 etherized mice were inoculated, in testing each serum-virus mixture, by dipping their noses in the inoculum contained in a slightly tilted small Petri dish. The mice were observed for 10 days; all dying were examined at postmortem; and on the 10th day, surviving mice were autopsied and the degree of pulmonary involvement was noted.

Because of the numbers of tests involved, all virus strains could not be studied at one time. The general plan followed, therefore, was to test all of the swine and rabbit sera against each of the strains of virus in turn. With the exception of strain 20, all of the swine influenza viruses were of roughly the same pathogenicity for mice, and the amount of virus administered in each test amounted to between 10 and 100 minimal fatal doses. Strain 20 possessed a slightly lower pathogenicity for mice, and the dilution used in the neutralization tests corresponded roughly to 10 minimal fatal doses. Two of the human influenza viruses, strains PR8 and WS, were of approximately the same pathogenicity as the majority of the swine strains, while the Oakham strain, at the time it was used, roughly corresponded in titer with strain 20 swine influenza virus. No effort was made to titrate the number of minimal fatal doses of virus more closely than by decimal dilutions. In each individual neutralization experiment 5 groups of control mice receiving virus mixed with normal rabbit or swine serum were included, and all of the mice in these groups succumbed of influenza during the 10 day period of observation.

RESULTS

The results obtained with convalescent swine sera are shown graphically in Chart 1 and those with immune rabbit sera in Chart 2. In

the two vertical columns to the left of each chart are listed the animals supplying the antisera together with the strains of virus against which the antisera were prepared.   The strain of virus used in neutralization

Serum		Strain of virus									
Swine No.	Immune to virus	S·15	S·20	S·23	S·24	S·28	S·BC	S·29	H·Oa	H·PR8	H·WS
84	S·15								▧	■	▧
85	S·15								▨	■	
9	S·20								▧	▧	▧
36	S·20								▧	■	
12	S·23								▧	■	
78	S·23	▧		▧	⊠	⊠		⊠		■	
95	S·24									■	
41	S·28	▧						▧	■	▧	
97	S·28								▨	▨	
69	S·BC								■	▨	
A 89	S·BC									▧	■
77	S·29									▧	▨
B 89	S·29			▧					■	■	▨
20	H·PR8	■	▨	■	■	■	■	■			
23	H·PR8	▪	■	■	■	■	■	■			▧
6	H·WS	■	■	■	■	■	■	■			
99	H·WS	■	▧	▧	■	▧	▧			▧	

CHART 1. Cross-neutralization tests in mice with convalescent swine serum.
■ All mice in the test died of influenza.   No neutralization.
▨ Mice survived but showed extensive lung lesions at autopsy on 10th day. Slight neutralization.
▧ Mice survived and showed only scant lung lesions at autopsy on 10th day. Partial neutralization.
□ Mice survived and showed no lung lesions at autopsy on 10th day.   Complete neutralization.
⊠ Not tested.

tests with the various sera is given at the top of each of the other vertical columns.

As shown in Chart 1 all swine convalescent sera, regardless of the

Serum		Strain of virus									
Rabbit No.	Immune to virus	S-15	S-20	S-23	S-24	S-26	S-BC	S-29	H-0ak	H-PR8	H-WS
44	S·15										
45	S·15										
63	S·15										
71	S·BC										
75	S·BC										
76	S·BC										
32	S·23										
74	S·23										
34	S·24										
78	S·24										
79	S·24										
29	S·28										
77	S·28										
50	S·29										
51	S·29										
53	S·29										
31	S·20										
55	S·20										
56	S·20										
24	H·PR8										
25	H·PR8										
46	H·PR8										
66	H·PR8										
68	H·PR8										
22	H·WS										
58	H·WS										
59	H·WS										
60	H·WS										

CHART 2. Cross-neutralization tests in mice with sera of immunized rabbits. Designation of results same as Chart 1.

strain of swine influenza virus from which the animals supplying the sera were convalescent, neutralized all strains of the swine influenza virus.   In like manner, the 3 human viruses tested were neutralized by the sera of swine recovered from infection with either the WS or PR8 strains of human influenza virus.   Between the human and the swine strains the serological relationship found to exist was variable; only one of the human virus antisera (swine 99) had any appreciable neutralizing effect on any of the swine viruses. In the reverse direction, however, most of the swine virus antisera partially neutralized the WS and Oakham strains.   The PR8 strain was neutralized partially by only one of the swine virus antisera. These findings taken alone would indicate that each strain of swine influenza virus was serologically like all of the other swine strains in the present experiments.   The 3 human viruses would also have to be considered alike on the basis of the results with the human virus antisera.   However, consideration of the neutralization tests with the human viruses and swine virus antisera makes it evident that the Oakham and WS strains behave quite differently from the PR8 strain, and it would seem that these two strains are immunologically more closely related to swine influenza virus than is the PR8 strain.   The important feature of the data given in Chart 1, so far as they concern the present experiments, is that no evidence is furnished to indicate serological heterogeneity among the 7 strains of swine influenza virus under study.

The results with virus-neutralizing rabbit sera recorded in Chart 2 are not as clear cut and constant as were those with swine sera. Among the swine influenza viruses, strains 15 and BC produced potent antibodies in rabbits both for themselves and for all heterologous swine strains as well, but were, as a rule, neutralized only partially or not at all by antisera prepared against the heterologous swine viruses.   Strain 20, on the other hand, was readily neutralized by sera prepared against all of the other swine strains and the PR8 human strain but itself produced antibodies poorly or not at all for the heterologous swine viruses.   Strain 29 resembled strain 20, though here one of the 3 rabbits used (rabbit 53) produced fairly good neutralizing antibodies for heterologous strains.   The 3 remaining swine strains resembled strains 15 and BC in that they produced antibodies in

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rabbits effective at least partially against all the other swine viruses but differed in that neutralization of the heterologous viruses was seldom complete as with the strain 15 and BC antisera. There are exceptions to this attempted classification, obvious from consideration of Chart 2. This suggests that at least some of the differences noted may be more dependent upon variations among the individual rabbits used than among the strains of swine influenza virus under study.

The rabbit antisera more effectively differentiated between the swine viruses and the WS and Oakham strains of human influenza virus than had the swine antisera. With the exception of PR8 antisera against strain 20, there was little cross-neutralization between swine and human strains. Furthermore, the rabbit antisera rather clearly differentiated between the PR8 and WS strains of human virus, something the swine antisera had failed to do.

DISCUSSION

It is difficult to reconcile the results obtained with swine convalescent sera and those obtained with sera of immunized rabbits as to their relative significance in denoting serological homogeneity or heterogeneity among the strains of swine influenza virus studied. If the results with swine convalescent sera were the only ones available, it would be simple to conclude that the 7 swine viruses were serologically alike and possessed the same general antigenic composition and pattern. If, on the other hand, only the results with sera of immunized rabbits were to be considered, it would be necessary to recognize the existence of antigenic variations among the swine influenza viruses. Thus, from the rabbit serum results, strains 15 and BC, which appear antigenically alike, differ from strains 20 and 29 in that they are not neutralized by antisera prepared against strains 20 and 29. Antisera prepared against 15 and BC do, however, neutralize strains 20 and 29. The remaining 3 strains lie intermediate between these two groups, though resembling strains 15 and BC most closely in their serological behavior. The classification which rabbit antisera seem to have made among the strains of swine influenza virus studied corresponds, in a way, with that into which Smith and Andrewes (7) grouped their human viruses. Strains 20 and 29 could be designated, according to this arrangement, as "specific" strains in

that they produce antibodies that are largely effective against only the homologous strains. Strains 15 and BC would correspond to Smith and Andrewes' "non-specific" or "master" strains, viruses which produce antibodies effective against the whole group of swine influenza viruses. The remaining viruses, strains 23, 24, and 28, would be classified as "intermediate" strains, though resembling the "non-specific" strains more closely than the "specific." There are, however, several individual exceptions to this rather general classification. For instance, rabbit 53, immunized with strain 29, developed antibodies that neutralized heterologous swine strains almost as broadly as sera prepared against 15 or BC. This serum also neutralized the WS strain human influenza virus completely, the only one of the anti-swine virus rabbit sera to be completely effective against any of the human viruses. In like manner, the antisera of rabbits 79 and 55 prepared respectively against strains 24 and 20 were unusual, when compared with antisera of other rabbits immunized with the same viruses, in their capacity to neutralize heterologous strains of swine influenza virus.

It is not believed that the various differences among the swine viruses, detectible by antisera prepared in rabbits, are due to differences in antibody titers of individual rabbit sera used, because frequently the differences are in the wrong direction to be accounted for in this way. Rather it would seem that rabbit antisera actually detect strain differences that are not reflected in convalescent sera of the natural host animal. Such differences are probably of no practical importance so far as the natural disease, swine influenza, is concerned and have an academic interest only in that they indicate a variation in the antibody response to the virus of a susceptible and a non-susceptible host.

Since, in the natural host of swine influenza, all strains of the virus give rise to an antibody response indicative of antigenic homogeneity, the question is raised as to whether the swine serum or the rabbit serum results should be more seriously considered in arriving at a decision as to whether the swine influenza virus strains studied are serologically alike or different. There can be no doubt that in rabbits the various virus strains give rise to antibodies with differing virus affinities. However, in the rabbit, swine influenza virus exhibits no evidence of pathogenicity and is probably not infective in the sense

in which that term is usually applied to indicate invasiveness and persistence of an infective agent in a susceptible host. In all probability, swine influenza virus acts in a manner analogous to that of any other invasively inert, antigenic substance in eliciting a specific response in rabbits. Thus if the swine influenza virus is antigenically complex, as Magill and Francis' (5) and Smith and Andrewes' (7) findings indicate the human influenza virus to be, then one might anticipate that the first antibody response of rabbits would be to the dominant or most readily accessible of the swine influenza virus antigens. In swine, on the other hand, where immunity follows actual multiplication of the virus within the host, invasion of susceptible cells by the virus, and finally, destruction or inactivation of virus at the time of recovery, one might expect an immunological host response to all of the various antigens comprising the virus. It seems entirely possible that the apparent discrepancies between the swine and rabbit serum findings may be accounted for by this difference in the mechanism whereby the virus-neutralizing antibodies are produced in a non-susceptible animal, the rabbit, on the one hand, and in a susceptible host, the swine, on the other. On such a basis, antisera prepared by the infection of swine with virus would be considered to reflect the entire antigenic content or composition of the virus, while antisera prepared by the injection of virus, infectively inert for rabbits, into these animals would be thought of as reflecting the arrangement, within the virus, of the components responsible for mouse pathogenicity. Such an explanation of the findings would orient the apparently discrepant results obtained with swine and rabbit antisera. The conclusion to be reached under this interpretation would be that the various strains of swine influenza virus studied are similar in their antigenic composition but that they vary among themselves either in the arrangement of their common antigenic components or in the situation, within the virus, of the components responsible for their mouse pathogenicity.

CONCLUSIONS

1. Cross-neutralization tests with sera from swine recovered from infection with swine influenza indicated the serological identity of 7 strains of swine influenza virus obtained from different sources.

2. Cross-neutralization tests with sera from rabbits, immunized

to swine influenza virus, exposed serological differences among the same 7 swine influenza virus strains. Two strains appeared to be serologically similar and were characterized by the ability to produce effective homologous virus-neutralizing sera which were, however, poor or ineffective against the heterologous virus strains. Two other strains were also serologically similar but produced antibodies effective not only against themselves, but against all heterologous strains as well. The remaining 3 strains were intermediate in their ability to produce heterologous virus-neutralizing antibodies.

3. The human influenza viruses included, especially strains WS and Oakham, were most effectively differentiated serologically from the swine influenza viruses by rabbit antisera.

4. The suggestion is advanced that swine antisera express the antigenic composition of the swine influenza viruses, while rabbit antisera reflect either their antigenic arrangement or the arrangement of the components responsible for their mouse pathogenicity. On this interpretation the 7 strains of swine influenza virus studied would be considered to have similar antigenic compositions but differing antigenic structures.

5. The serological differences among strains of the swine influenza virus, detectible by rabbit antisera, are probably of no practical significance so far as the natural disease, swine influenza, is concerned.

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AMOUNT AND DURATION OF IMMUNITY INDUCED BY INTRADERMAL INOCULATION OF CULTURED VACCINE VIRUS

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Jennerian prophylaxis in man by means of intradermal inoculation of cultured vaccine virus was described (1) in 1935. Since then this type of prophylaxis against smallpox has been used by us, by many private physicians, and by physicians in a number of clinics. For the last 3 years the virus employed has been from generations 50 to 170 of the "second revived" strain of cultured vaccine virus, dried from the frozen state in the presence of gum acacia and sealed *in vacuo* (1, 2). This strain (3) was originally derived from calf lymph vaccine virus supplied by the New York City Board of Health in 1931 and has been propagated since then by serial transfers in a medium consisting of Tyrode's solution and minced chick embryo tissue. Intradermal inoculation of the virus in rabbits reveals that it maintains a uniform potency during continued cultivation. Moreover, the lesions produced in rabbits are less severe than are those caused by other strains of vaccine virus.

When inoculated intradermally in susceptible persons, cultured vaccine virus produces a high percentage of positive reactions. The percentages reported by those to whom the virus has been dispensed have ranged from 80 to 100; it is probable that an average of over 90 per cent has been obtained in a minimum of 6000 to 7000 intradermal vaccinations. In our experience with more than 200 primary vaccinations the incidence of "takes" has been 100 per cent. Typical positive reactions appear as small red papules on the 4th to 9th day after inoculation. Erythema and induration increase until the lesions are 2 to 4 cm. in diameter at their height 4 to 6 days later. Erythema disappears rapidly although induration may persist 4 or 6 weeks. If

the inoculation is made properly, no vesicle forms to leave a scar. Those who have observed or experienced reactions produced in this way have been pleased by the lack of accompanying constitutional symptoms and the absence of open sores.

Knowledge of the amount and duration of immunity to smallpox induced by the intradermal injection of cultured vaccine virus is of cardinal importance. However, no opportunity of observing the incidence of smallpox in a group of individuals vaccinated in this manner has arisen. Nevertheless, from experience it is known that the efficacy of any type of vaccination against smallpox can be tested by revaccination with a potent calf lymph vaccine virus. In spite of this fact, reports of the effect of primary vaccination with cultured vaccine virus on subsequent revaccination with calf lymph virus have been few. In 1935 (1) we described the results obtained in the revaccination of 7 persons who had been successfully vaccinated with cultured virus 13 days to 7 months previously. Of these, 6 were immune to New York City calf lymph virus, while 1, after an interval of 7 months, responded with an accelerated take. In 1937 (4) we conducted revaccinations on a small group of children who had been vaccinated with cultured vaccine virus 1 month to $2\frac{1}{4}$ years previously. In 6 of 14 such children the response to calf lymph virus was that of an accelerated take, *i.e.*, vesicles formed and the reactions were not at their maximum until the 5th or 6th day.

During the last year and a half we have studied a large group of children in order to obtain more complete information concerning the amount and duration of immunity produced by cultured vaccine virus against the New York City calf lymph strain of virus. The results of the study will be reported at this time. In addition, information regarding the immunity produced by cultured virus against other strains of calf lymph vaccine virus, as well as a consideration of the effect that differences in the manner of performing primary inoculations with cultured virus have on subsequent immunity, will be presented.

Methods

At the Rockefeller Hospital there is little opportunity of performing primary vaccinations, in consequence of which it has not been possible in this clinic for us to observe the response of a large number of children to revaccination. However,

at the Children's Prophylactic Clinic of the New York Hospital, cultured vaccine virus supplied by us has been administered intradermally for several years. The facilities and records of this clinic were made available to us through the courtesy of Dr. Samuel Levine and Dr. Parker Dooley.

Children were selected in whom a positive primary vaccination with cultured virus had been observed and recorded and in whom no further prophylaxis against smallpox had been carried out. Each child was revaccinated with New York City Board of Health calf lymph vaccine virus applied to a linear scratch ⅛ inch in length. In addition various groups received on the opposite limb commercial calf lymph virus A or B applied to a linear scratch. All virus used was received fresh each week from the place of preparation and was stored at 0°C. before use. A single observation on the 5th day after revaccination has, as a rule, been all that could be made; the few that could not be seen on the 5th day were seen between the 4th to 7th days.

The time at which a reaction to vaccine virus is at its maximum and not the size of the lesion is considered to be the correct index of susceptibility (5). Reactions are usually classified as no reaction, immune reaction, accelerated take, primary take. Due to the impossibility of making frequent observations on the revaccinated children only two types of reaction are recorded, namely, immune reactions and accelerated takes. Immune reactions are those which showed on the 5th day only a small papule or some evidence that a mild response to inoculation had been present. The children who showed at this time no evidence that the virus had been effectively introduced into the skin were excluded from consideration. Thus, a few rapid immune reactions may have been missed, but the number was not great enough to affect significantly the results of the study. Accelerated reactions comprise those which on the 5th day showed the presence of a vesicle surrounded by a zone of erythema. The use of vesicle formation as one of the criteria for classification has insured the inclusion in the group of accelerated takes of even the mildest of this kind of reaction, viz., one which heals without the formation of an enduring scar. It is possible that some of the reactions may have been in an early stage when the results were recorded and that the time at which the maximum sizes were reached approached closely the time at which primary takes would have been at their height. However, from observation of some children later than the 5th day after inoculation and from the size of resultant scars which we have seen, we believe that most of the accelerated reactions were correctly classified.

Results of Revaccination with New York City Calf Lymph Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Vaccine Virus

331 children who had received one inoculation of cultured virus resulting in a primary take 1 month to 3 years and 9 months previously were revaccinated with New York City calf lymph virus (Table I).

Of these, 82 or 25 per cent responded with immune reactions, while 249 or 75 per cent showed accelerated takes. Most of the accelerated reactions were mild in character, presenting a small vesicle surrounded by a zone of erythema 0.5 to 1 cm. in width. Frequently on the 5th day the contents of the vesicles were drying or inquiry revealed that the lesions had been larger or as large on the preceding day. Children responding in this manner did not present the usual symptoms and signs that as a rule accompany primary vaccination with calf lymph. There were others, however, in whom the lesions presented no signs

TABLE I

Results of Revaccination with New York City Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Virus

Number of children revaccinated	Time between primary and secondary vaccinations	Immune reactions		Accelerated takes	
		Number	Per cent	Number	Per cent
39	1–6 mos.	13	33	26	67
76	6 mos.–1 yr.	25	33	51	67
185	1–2 yrs.	37	20	148	80
31	2+ yrs.	7	23	24	77
Total 331		82	25	249	75

Distribution of age at time of primary vaccination similar for all groups.

of regression on the 5th day and who experienced later fever and lymph gland enlargement accompanying the presence of a central pustule in a zone of erythema and induration of considerable extent. Nevertheless, healing of these lesions was rapid and the scars which resulted were small and superficial.

Analysis of the data obtained in this group of 331 children revealed that the proportion of immune individuals was fairly constant and bore no relation to the interval which had elapsed between the primary vaccination with cultured virus and revaccination with calf lymph (Table I). It is true that the percentage of accelerated reactions was slightly higher in the children revaccinated after 1 year and that the more severe reactions were observed in this group, but the figures obtained give little indication that susceptibility to calf lymph virus increased with the lapse of time within the limits of the observations.

It is known that infants shortly after birth (6) are somewhat resistant to infection with vaccine virus. Furthermore, it has been demonstrated (6) that such infants after a successful vaccination rapidly lose their immunity, many being fully susceptible a year later. The results obtained in our group of 331 children, the majority of whom were first vaccinated between the ages of 6 months and 1 year, indicate that the differences in the age at which the primary vaccinations were performed had no influence on the proportion of children

TABLE II

Results of Dermal Revaccination Made with New York City Vaccine Virus to Ascertain the Duration of Immunity Produced by One Successful Intradermal Inoculation of Cultured Virus in Relation to the Age of Children at Time of Primary Vaccination

Number of children revaccinated	Age when first vaccinated	Immune reactions		Accelerated takes	
		Number	Per cent	Number	Per cent
69	6-9 mos.	15	22	54	78
98	9 mos.-1 yr.	29	30	69	70
55	1-2 yrs.	14	25	41	75
35	2-3 yrs.	7	20	28	80
53	3-5 yrs.	11	21	42	79
21	5+ yrs.	6	29	15	71
Total 331		82	25	249	75

Distribution of interval of time between primary and secondary vaccinations similar for all groups.

who retained complete immunity during the period of observation (Table II).

Results of Revaccination with Commercial Strains of Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Vaccine Virus

Reports in the literature concerning the duration of immunity in children to vaccine virus are conflicting. Moreover, in attempting to evaluate the results of different workers, one is confused by a lack of uniformity in classification or description of the type of reaction produced by revaccination and by the fact that the relative potency

of the viruses used for the primary vaccinations and revaccinations was either not known or not stated. A mildly acting virus does not always fully protect for a great length of time against a virulent strain, and results obtained by revaccination with a mild strain may not parallel those secured by revaccination with a potent virus. The New York City vaccine virus is a strain of high uniform potency. Consequently, it seemed of interest to compare the results obtained by

TABLE III

Results of Dermal Revaccination with Commercial Strains of Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Virus

Number of children revaccinated	Revaccinated with New York City virus		Revaccinated with commercial virus A		Revaccinated with commercial virus B	
	Per cent immune reactions	Per cent accelerated takes	Per cent immune reactions	Per cent accelerated takes	Per cent immune reactions	Per cent accelerated takes
78	22	78	72	28		
82	35	65			55	45

Distribution of age at time of primary vaccination and interval of time between primary and secondary vaccinations similar in both groups.

means of its use in the revaccination of children with those secured by revaccination with other strains of calf lymph vaccine virus. Two commercial preparations of calf lymph virus, A and B, were chosen because they are products widely used in the United States. 78 of the 331 children who were revaccinated with New York City calf lymph received at the same time an inoculation with commercial lymph A; a second group of 82 children received in addition to New York City virus an inoculation of commercial calf lymph B. Of the 78 children, 17 or 22 per cent responded with immune reactions to New York City virus, while 56 or 72 per cent responded with immune reactions to calf lymph A; of the 82 children, 29 or 35 per cent were immune to New York City virus, while 45 or 55 per cent responded in that manner to calf lymph B. These figures, recorded in Table III, show discrepancies that may result from the use of different strains of virus.

Effect of Differences in Primary Inoculation of Cultured Vaccine Virus on Subsequent Revaccination with New York City Board of Health Calf Lymph

At this point it seemed of value to learn whether the administration of large doses of the mildly acting cultured virus or the production of 2 intradermal lesions at the same time would influence the resultant immunity. Accordingly, a group of children at the New York Hospital Clinic were given 2 intradermal inoculations, one in each arm or thigh, of cultured vaccine virus. There were no untoward results. The simultaneous evolution of 2 intradermal vaccinal lesions produced by cultured vaccine virus apparently caused the children no more inconvenience than that evoked by a single reaction. From this group of children, 66, whose records showed that they had had 2 successful simultaneous primary vaccinations, were revaccinated dermally with New York City calf lymph 2 to 6 months later. Of the 66 children, 18 or 27 per cent responded with immune reactions, while 48 or 73 per cent showed accelerated takes. Comparison of these figures (Table IV) with those obtained in the group of 331 (Table I) who received only a single injection of cultured virus for primary vaccination shows that the introduction of a double amount of this virus and the production of 2 primary lesions instead of one did not alter the percentage of children who retained for 6 months complete immunity to the New York City calf lymph.

As stated previously, the virus which has been used during the last 3 years was obtained from the 50th to the 170th culture generations of the "second revived" strain. This virus was selected for human inoculation because it produced mild reactions and maintained a constant potency for man and rabbit. However, from previous experience (2) with the original strain we had noted that the infectivity of the virus diminished on repeated passage in culture and that a change in the character of the lesions produced by it in rabbits also occurred during serial transfer of the virus in the medium used. It occurred to us that a gradual change might have taken place in the "second revived" strain, less marked than that noted in the original one but still great enough to influence the amount of protection produced against a highly potent strain of vaccine virus or against

smallpox. Therefore, it seemed important to determine whether continued cultivation of the "second revived" strain had resulted in a loss of some of its antigenicity essential for the development of a lasting immunity. In order to make this determination, cultured virus from the 20th to the 30th generations of the "second revived" strain was prepared for human inoculation and tested in rabbits and in man.

Intradermal inoculation of the virus in rabbits revealed that the infectivity or titer was essentially the same as that of generation 50 to 170, but the lesions produced by the early generations were more edematous and more hemorrhagic and necrotic than were those

TABLE IV

Results Obtained by Dermal Revaccination with New York City Vaccine Virus of Children Who Had Been Primarily Vaccinated Intradermally in Several Different Ways with Cultured Virus

Type of primary inoculation	Number of children revaccinated	Number of immune reactions	Per cent of immune reactions	Number of accelerated takes	Per cent of accelerated takes
Single inoculation with virus from 50–170th culture generation	331	82	25	249	75
Double inoculation with virus from 50–170th culture generation	66	18	27	48	73
Single inoculation with virus from 20–30th culture generation	54	33	61	21	39

produced by later generations of the active agent. Each of 7 volunteers was inoculated intradermally with 0.1 cc. of a 1:10 dilution of the virus. The lesions produced by this material were larger and more severe than those caused by virus from later generations; however, the reactions were not severe enough to cause anxiety regarding the use of the material. Consequently, a study of immunity produced by it was carried out in a group of children at the New York Hospital Prophylactic Clinic. 54 children were inoculated intradermally with cultured virus from generations 20 to 30; 2 to 6 months later they were reinoculated dermally with the New York City calf lymph virus. Of the 54 children, 21 or 39 per cent responded with accelerated takes, while 33 or 61 per cent showed immune reactions (Table IV). The accelerated takes were mild and healed quickly leaving only small superficial scars. These results are significantly different from those

obtained in children primarily vaccinated with virus from culture generations 50 to 170.

DISCUSSION

Vaccination against smallpox by means of dermal application of potent calf lymph vaccine virus is efficacious. Nevertheless, considerable inconvenience and, at times, danger are associated with this type of vaccination which always leaves an ugly scar. In view of these facts, many people in the United States have never been vaccinated. To overcome opposition to vaccination certain health officials minimize the inconvenience and speak of the scar as a "badge of health." Thus, they leave the impression that a person with a scar is protected against smallpox and is not in need of revaccinations at regular intervals. As a matter of fact, all that a scar indicates is that an individual has been vaccinated; it does not show that the person is immune to smallpox. That can be determined only by the results of revaccination with a potent calf lymph virus. Moreover, the longer a person has gone since a primary vaccination, the more likely is he to have lost protection and the greater is his need of revaccination. Some individuals lose immunity more rapidly than do others; this appears to be particularly true of young children and infants. Therefore, revaccinations should be made at regular intervals; in the presence of smallpox epidemics revaccinations should be made regardless of when primary vaccinations or revaccinations were performed.

With the idea that vaccination against smallpox can be made a safer procedure, that mutilation is not an essential feature of the procedure, and that a scar gives the individual as well as the health officer a false sense of security, we undertook a number of years ago to prepare a vaccine virus that could be used in a manner less objectionable than that now employed with calf lymph virus. From the results obtained by us in the use of cultured vaccine virus for Jennerian prophylaxis in man we have become convinced that the ideas which prompted the work are entirely sound.

Continued cultivation of vaccine virus in the medium used by us has brought about a qualitative change in the active agent which makes it possible to introduce considerable amounts of the material intradermally without danger and inconvenience to patients. It has been found, however, that the amount of immunity produced by the

cultured virus, as tested by means of a highly potent calf lymph vaccine virus, would probably not be considered sufficient for complete protection against smallpox.   On the other hand, when commercial vaccines widely used in the United States are employed for testing the immunity induced by the cultured virus, the results might be considered satisfactory.   At present we are suggesting that primary vaccinations be made intradermally with our cultured virus and that revaccinations be made dermally six months to a year later by means of a potent calf lymph virus.   In this way vaccinated individuals will not become sick and will not be subjected to the dangers associated with primary vaccinations with calf lymph virus, but will obtain a solid and lasting immunity to smallpox.   It is possible and highly probable that a cultured virus can be developed which will be suitable for intradermal use and will not require prompt dermal revaccinations with a potent calf lymph virus to produce an enduring immunity. These are matters for future investigation.

CONCLUSIONS

Continued cultivation of vaccine virus in a medium consisting of minced chick embryo tissue and Tyrode's solution has resulted in a virus qualitatively changed to such an extent that considerable amounts of it can be injected intradermally into human beings without danger or inconvenience.

Individuals who are vaccinated intradermally with the cultured virus should be revaccinated dermally six months to a year later with a potent calf lymph virus in order to obtain a satisfactory immunity to smallpox without being subjected to the dangers and inconvenience associated with primary vaccinations with calf lymph virus.

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PROTECTIVE ANTIBODIES IN THE SERUM OF SYPHILITIC RABBITS*

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PLATE 44

It has been known for many years that one attack of syphilis confers an increased resistance to a second attack. Persons in whom characteristic generalized lesions of early syphilis develop usually recover, even in the absence of specific treatment, and rarely again develop widespread lesions of the skin or mucous membranes or bones. The presence of a relative immunity in man following infection with *Treponema pallidum* has also been demonstrated by reinoculation. Most of the available information on immunity in syphilis has been gained, however, from a study of the disease in animals.

The work of Neisser and his associates on monkeys and the higher apes, and the studies of Uhlenhuth, Mulzer, Brown, Pearce, Chesney, Kolle, Frei, and Breinl, to mention only a few, on rabbits, have provided a fairly clear picture of the degree of resistance to reinoculation that is developed by animals infected with *T. pallidum*, the distribution of the resistant state among various tissues of the animal's body, and the time relationships concerned in the evolution of this immunity. In brief, these studies show that in rabbits immunity is acquired slowly and does not reach its full development for a number of months after infection; that it is not sufficient completely to rid the body of the infectious agent; that it tends to be strain specific; and that the acquired resistance of the animal may be overcome by conditions which favor the infecting organism as against the host. The experiments bearing on this subject were summarized by Chesney in a comprehensive review published in 1927 (1), while the more recent work has been

reviewed by Harrison (2), Topley and Wilson (3), and Zinsser, Enders, and Fothergill (4), among others.

Included among the studies on the general subject of immunity in syphilis have been many designed to demonstrate the presence of antibodies specific for *T. pallidum* in the blood serum of syphilitic human beings or animals. In general these efforts have met with failure, or, at best, the results have been equivocal. Complement-fixing antibodies and precipitins are commonly present in the serum of syphilitic patients, but the demonstration of these substances rests on tests which superficially at least, are biologically non-specific. A number of workers, including Hoffman and von Prowazek (5), Zabolotny and Maslakowitz (6), Touraine (7), and Blum (8), obtained results suggesting the presence in the serum of man or animals of either agglutinins or treponemicidal substances for virulent *T. pallidum*, but these experiments were far from conclusive and numerous other investigators, including Landsteiner and Mucha (9), Zinsser, Hopkins, and McBurney (10), and Beck (11), have been unable to demonstrate such antibodies. A large number of experiments have also been done with strains of treponemes, supposedly *T. pallidum*, that have been cultivated on artificial media. In general, these studies show that, upon injection of culture treponemes, animals develop agglutinins and treponemicidal substances against these spirochetes, but not against virulent *T. pallidum* derived from man or animals (1). Likewise, the serum of syphilitic persons or animals usually fails to agglutinate or kill culture treponemes, although Zinsser, Hopkins, and McBurney (12) noted that serum from syphilitic rabbits agglutinated these organisms in higher dilution than did the serum from normal rabbits. Moreover, serum from patients with tertiary syphilis showed greater agglutinating power than serum from normal persons. In recent years, however, considerable doubt has been thrown on the identity of culture treponemes and *T. pallidum*, and the failure of syphilitic serum to act upon culture treponemes may not be of great significance.

The studies of two groups of investigators, however, are worthy of special note, since they are not in accord with the majority of studies on the subject of humoral immunity in syphilis. In 1921 Eberson (13) recorded experiments in which serum from persons with late syphilis and from rabbits with syphilitic infection of 6 months' duration or longer, when combined with virulent *T. pallidum* and incubated for 2 hours at 36°C., completely protected rabbits against infection when the mixture was inoculated intratesticularly. Serum from normal persons and persons with early syphilis, and serum from normal rabbits and rabbits infected for less than 6 months exerted no such protective action, for rabbits inoculated with the incubated mixtures developed characteristic evidence of syphilitic infection. Unfortunately, this author failed to give certain important details of these experiments, but apparently no exceptions were noted to the general results as stated. Moreover, no other investigator has succeeded in obtaining such complete protection of normal rabbits with serum from persons with late syphilis and with serum from rabbits infected for 6 months or longer.

More recently, Tani and his coworkers have published a series of papers on the

question of humoral immunity in syphilis. Tani, Saito, and Funada (14) tested the serum of two rabbits with syphilitic infection of long duration (231 and 295 days, respectively) by combining the serum with emulsions of virulent *T. pallidum* in serial dilutions, incubating the mixture for varying periods of time, and inoculating these mixtures intracutaneously in normal rabbits. As controls, serum from normal rabbits, plus spirochete emulsion treated in the same manner, were inoculated intracutaneously in the same rabbits as was the serum from the syphilitic animal. Evidence of the protective action of the syphilitic serum was manifested by a longer incubation period and smaller size of the lesions developing at the site of inoculation of syphilitic serum-spirochete emulsion, compared with those of the controls. In a subsequent paper Tani and Ogiuti (15) reported the results of similar tests made on serum from 6 rabbits infected from 18 to 79 days, and the serum of 7 patients with secondary syphilis and 3 with general paresis. On the whole the serum from the syphilitic persons and animals showed slightly greater protective power than did normal serum, but the differences were not marked. The technique used in these experiments was rather complicated and the results are not altogether clear cut.

Of considerably greater interest, however, are the parabiosis experiments performed by Tani and Aikawa (16). These investigators parabiosed 42 pairs of rabbits in which one of the pair had been infected with syphilis from 99 to 459 days, and the other from 9 to 94 days. None of the former had active syphilitic lesions and all were probably immune; all of the latter had active syphilitic lesions at the time of the parabiosis operation. By tests with trypan blue, and neoarsphenamine, and by other methods it was shown that exchange of body fluids began about the 4th day and free circulation between the parabiosed animals was usually established by the 10th day. Among 35 pairs surviving for 9 days or longer, all but 7 showed definite healing of the active lesions, as manifested by decrease in induration and, frequently, the disappearance of spirochetes from the lesions. Of 14 control pairs in which an animal with active lesions was joined to a normal rabbit and observed for 9 days or longer, evidence of healing was manifest in only 2. The results of these experiments seem to be quite definite and, if confirmed, to prove the existence of humoral antibodies in syphilitic rabbits. Moreover, these antibodies appear to play an important rôle in the defense mechanism of the host.

This paper will present the results of experiments designed to show the existence of protective antibodies in the serum of rabbits resistant to reinfection, and to describe a technique by which this protective action of the serum may be demonstrated.

Experimental Method

In developing a technique to show the protective power, if any, of syphilitic serum, consideration was given to the idea that, at best,

the antibody titer of serum from rabbits immune to syphilis was probably low, and that any test designed to show the presence of these antibodies must be sufficiently finely adjusted to bring out the small differences that probably exist between the serum of normal and syphilitic animals or human beings. To this end many preliminary experiments were made, and the technique of the test outlined here represents the one which is in use at the present time, although many of the experiments recorded were not performed exactly with this present technique.

In brief, 9 parts of whole serum are combined with 1 part of spirochete emulsion, the mixture is incubated at 37°C. for 6 hours, and then is inoculated intracutaneously on the backs of normal rabbits. The rabbits are observed at daily intervals and the incubation period and size of the resulting syphilitic lesions are noted. The details of the test and the experimental method employed in these studies are given in full below.

Serum Tested.—The serum tested was obtained from rabbits which had been infected with the Nichols strain of *T. pallidum* at least six months prior to bleeding. No antisyphilitic treatment had been given. From the experiments of others, to which reference has already been made, it could be assumed that the rabbits would, upon reinoculation, prove to have at least a chancre immunity and probably to be entirely resistant to reinfection. The presence of chancre immunity was actually proved in a number of these animals by reinoculation of an homologous strain of *T. pallidum.* As controls, serum from 2 or 3 normal rabbits, bled at the same time as the test animals, was pooled and subjected to the same procedures as serum from the test animals. The whole blood was placed in the ice box overnight and the serum pipetted off the following day. It was then tested on that day, or else frozen in solid carbon dioxide and 95 per cent alcohol at −78°C. until ready to be used. The serum was not heated above 37°C. before testing.

Spirochete Emulsion.—Normal rabbits were inoculated in both testes with the Nichols strain of *T. pallidum.* When the resulting orchitis was fully developed the testes were excised, weighed, ground in a mortar to which a small amount of sand had been added, and diluted with ordinary culture broth in an amount in cubic centimeters corresponding to the weight of the organs in grams. The suspension was centrifuged in order to throw down the large tissue particles. The supernatant fluid, which was ordinarily rich in motile *T. pallidum*, was termed a 50 per cent emulsion. The strength of such emulsions as judged by number of spirochetes or its infectivity for rabbits, varies, of course, with each batch, and designation in terms of percentages is meant to carry only an approximate estimate as to its virulence.

Occasionally this emulsion was used in the protection test on the day it was prepared, but the usual procedure followed was to distribute the emulsion in 1 or 2 cc. vials, tightly stopper the vials, and freeze them in solid carbon dioxide and

alcohol at a temperature of −78°C., until needed. As reported in a previous paper, the virulence of *T. pallidum* can thus be preserved essentially unchanged for periods of months or even years (17). Graduated dilutions of this emulsion were then combined with normal rabbit serum in the same proportion as that used in the test and titrated by inoculating the mixture intracutaneously in normal rabbits. It was thereby possible to determine approximately the least amount of spirochete emulsion which would fairly regularly produce a lesion at the point of inoculation within a period of 4 weeks. This may be termed the "minimal chancre dose," and ordinarily varies from a final dilution of 1 to 5 per cent when 0.1 cc. is the amount inoculated, and the period of incubation is 6 hours at 37°C.

Proportion of Spirochete Emulsion to Serum.—Different proportions of spirochete emulsion to serum have been used in the experiments reported here. As the test is now being performed a proportion of 1 part of spirochete emulsion to 9 parts of serum is used, but proportions of 1 to 3 and 1 to 4 have also been employed. It is probable that an excess of serum is being used; this point requires further investigation.

Incubation of Mixture.—The serum-spirochete emulsion is thoroughly mixed and placed in the incubator at 37°C. for 6 hours. Care should be taken to see that the temperature of the incubator does not rise above this point. As will be noted below, in some experiments shorter periods of incubation were used. At the expiration of the period of incubation the serum-spirochete emulsions are again mixed and cultures made on blood agar plates in order to rule out the presence of contaminating bacteria. The mixtures are now ready for inoculation.

Inoculation of Mixtures.—In the preliminary experiments normal rabbits belonging to the ordinary laboratory stock were used. In the latter experiments all test rabbits were male animals of the Dutch Belt variety, all were obtained from one dealer supplying a highly inbred stock, and all animals were young adults of approximately the same age. The backs of the animals were prepared for inoculation by closely clipping the hair with electrically operated clippers.

Inoculations are made with tuberculin syringes and 27 gauge needles. The back of each rabbit is divided into 4 areas, and inoculations are made intracutaneously in 6 sites of each area, 0.1 cc. of the mixture being injected at each site. 4 animals are customarily inoculated with each mixture. If syphilitic lesions develop at each site of an area, a characteristic pattern is noted. This pattern assumes importance when the lesions begin to develop, for it aids materially in distinguishing between syphilitic lesions and non-specific lesions which are not infrequently encountered. The pattern of beginning syphilitic lesions is unmistakable, however, and greatly facilitates accurately establishing the incubation period of the lesions. A diagram of the rabbit's back showing the areas and sites of inoculation is presented in Text-fig. 1.

Reading the Test.—All animals were commonly examined daily during the period of observation. Frequently on the day following inoculation a slight non-specific reaction, characterized by erythema

and slight edema at the sites of inoculation, was noted. This usually subsided by the 2nd or 3rd day, and the inoculated areas remained negative until the beginning of syphilitic lesions in one or another area. As will be noted later, the incubation period of these lesions varied considerably, depending on a number of factors. As the syphilitic lesions develop at the site of inoculation an effort is made to record their relative size for comparative purposes. Characteristically, the lesions begin as small erythematous spots and progress, within a period of a week or 10 days, to typical indurated papules closely resembling the hunterian chancre considered to be so characteristic of primary syphilis in man. Motile *T. pallidum* were demonstrated in many of the lesions. Numerous ways of giving a numerical expression to these lesions have been tried, and the most satisfactory seems to be to record the relative size of the lesions in each animal in terms of 1 plus, 2 plus, 3 plus, or 4 plus (+, ++, +++, ++++). The sum of these numbers in any one area is then used to indicate the size of the lesions in that area. For example, if a well developed chancre is present at each of 6 sites in one area these would be designated 4 plus lesions and the total of these figures would be 24. In another area showing 1 plus lesions the total size would be read as 6. Actual measurements of the lesions have been made, but because the lesions are three dimensional this method often gives a less accurate picture than the one just described.

TEXT-FIG. 1. Schematic representation of rabbit's back showing areas and sites of intracutaneous inoculation of serum-spirochete mixtures.

Schematic drawings of the lesions have been made twice weekly during the period of observation, and these provide the most accurate picture of all. The actual area of the lesion is drawn and the degree of induration or elevation is represented by crossed lines; the closest cross-hatching representing the greatest elevation. Drawings made from four animals are reproduced in Text-fig. 2.

Duration of Observation.—In the experiments reported here, no uniform period of observation was employed, and as a rule animals were observed until the first lesions had apparently reached their

maximum development. In the experiments still in progress, a uniform practice has been adopted of observing the animals 7 days after the first pattern of syphilitic lesions is noted. The size of the lesions recorded in the following experiments represents the readings made on the last day of observation or on the day of maximum development of the lesions, in case they had begun to subside before the animal was discarded.

Factors Affecting the Test.—Among the most important variables affecting the test was the virulence of the spirochete emulsion. In the latter experiments, however, this factor was controlled by using material which had been previously tested and preserved by freezing. Another important variable was the temperature of the room in which the inoculated rabbits were maintained. In general, the higher the temperature the longer the incubation period. This effect of temperature is well known, and is a factor which must always be reckoned with in studies on experimental syphilis. It is possible that protection tests, such as described here, cannot be successfully performed at temperatures usually encountered during the summer months.

Another variable factor is the susceptibility of individual rabbits to syphilis. The vast majority of animals, particularly from so uniform a stock as now in use here, react in much the same manner to the same inoculation. In every large series, however, there will be an occasional animal that seems to be unusually susceptible, as indicated by a much shorter incubation period of the syphilitic lesions and by the larger size of the lesions. Likewise, an occasional rabbit is encountered that seems to be unusually resistant to syphilitic infection, as manifested by a delayed appearance and slow development of the lesions. Rarely an animal will not develop any lesions at all following inoculation of the same mixtures that give rise to characteristic syphilitic lesions in a large number of other rabbits. It is for this reason that normal sera used as controls in these experiments were commonly obtained from two or more rabbits.

EXPERIMENTAL RESULTS

Preliminary Experiments.[1]—As a starting point for the investigation of this problem a number of rabbits that had been infected with

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[1] Some of the preliminary experiments were performed in the laboratory of the Syphilis Division of the Johns Hopkins Medical School, which is under the direc-

T. pallidum for from 6 months to 1 year were selected. All had had extensive syphilitic lesions, which at the time of these experiments were apparently inactive. No antisyphilitic treatment had been given. The animals were reinoculated intracutaneously with a homologous strain of treponeme and were shown to be refractory to reinoculation. Serum from these animals was tested numerous times along with serum from normal rabbits. During these preliminary experiments one or another of the various procedures in the test was varied in an effort to find the combination that seemed to yield the best results. Tests were made with varying dilutions of spirochete emulsion, with different amounts of serum, and using varying periods of incubation. In none of these experiments was the serum heated above 37°C. Several other experiments were made to test the effect of heating the serum to 56°C. in order to inactivate the complement but these will be referred to later.

The results of these preliminary experiments will not be given in as much detail as will those of the subsequent experiments, but a summary of the results is shown in Table I. Each separate test with serum from immune rabbits was controlled by inoculating mixtures of normal serum which had been subjected to exactly the same procedures.

The results with immune serum-spirochete mixtures obtained on intracutaneous inoculation of the test rabbits have been designated "definite protection," "questionable protection," or "no protection," in relationship to the results obtained in the same animals with simultaneous inoculation of normal serum-spirochete mixtures. A clearer idea of how these designations were made can be obtained by referring to the more detailed accounts of the experiments presented below.

Without regard to the details of the tests, it is noted that of 56 areas inoculated with immune serum-spirochete mixtures 42 showed evidence of definite protective action on the part of the immune serum, as compared with the areas inoculated with normal serum-spirochete mixtures. In 10 areas questionable evidence of protection was noted, and in only 4 areas was there no evidence of protection.

Of the animals tested, the original inoculation had been intratesticular in rabbits 46-75 and 46-87, and intracutaneous in rabbits 47-70, 47-72, and 47-73. It is of interest that, in each of three series of

TABLE I

Results of Protection Tests Made with Serum from Rabbits Immune to Syphilis
Preliminary Experiments

Source of "immune" serum tested		Details of test			Results of test in inoculated rabbits			
						Number showing		
Rabbit No.	Dura-tion of infection	Dilution of spiro-chete emulsion	Propor-tion of spiro-chete emulsion to serum	Incubation of mixture Temperature and time	Number inocu-lated	Definite protec-tion	Ques-tionable protec-tion	No protec-tion
	mos.	*per cent*						
46-75	12	10	1:3	37°C. 30 min.	2	2	0	0
"	12	1	"	" " "	2	2	0	0
"	12	0.1	"	" " "	2	2	0	0
"	12	20	"	" " "	3	3	0	0
"	18	50	"	" 3 hrs.	4	3	1	0
46-87	16	"	"	" " "	4	3	0	1
47-70	11	"	"	" " "	4	3	1	0
47-72	11	"	"	" " "	3	0	2	1
47-73	11	"	"	" " "	3	3	0	0
46-75	18	1	"	" " "	3	2	1	0
46-87	16	"	"	" " "	3	3	0	0
47-70	11	"	"	" " "	4	4	0	0
47-72	11	"	"	" " "	3	1	0	2
47-73	11	"	"	" " "	2	1	1	0
46-75	19	10	"	" " "	3	3	0	0
46-87	17	"	"	" " "	3	3	0	0
47-70	12	"	"	" " "	3	2	1	0
47-72	12	"	"	" " "	3	1	2	0
47-73	12	"	"	" " "	2	1	1	0

tests, serum from rabbit 47-72 tended to exhibit less protective power than serum from the other animals.

With reference to the details of the technique of the test, the most definite finding drawn from the preliminary experiments was that the virulence of the spirochete emulsion was probably the most important

feature. Emulsions which were too virulent tended to obliterate differences in the incubation period, while if the spirochete emulsion was of relatively low infectivity even the normal serum-spirochete emulsions failed to produce lesions at the site of inoculation. Because of these findings a spirochete emulsion which had been previously tested for virulence and preserved by freezing was employed in subsequent experiments.

Effect of Inactivating Complement.—During the course of the preliminary experiments samples of sera from the same animals were tested after having been heated for 30 minutes at 56°C. in order to inactivate the complement. Altogether, 11 sera from 5 immune rabbits were tested by the same technique as used in the foregoing experiments. All animals had been infected by intratesticular inoculation from 7 to 12 months prior to bleeding. Of a total of 42 areas injected with immune serum-spirochete mixtures, 13 showed definite evidence of the protective action of the serum as compared with the areas in which normal serum had been injected, 12 showed questionable evidence of protection, and in 17 there was no evidence of protection. These results are distinctly poorer than when sera from the same animals were tested without heating above 37°C., and they suggest that the presence of active complement is favorable to the demonstration of the protective action of immune serum. To what extent active complement was present in the testicular emulsion is not known, but it is possible that enough was available to satisfy the requirements of the test. This point needs further study.

Experiment 1.—The four sera used in this experiment were obtained from 2 immune animals, 46-75 and 46-87, and 2 normal rabbits, normal A and normal B. One part of a 25 per cent testicular emulsion containing numerous treponemes was added to 4 parts of serum and the mixture incubated for 6 hours at 37°C. At the end of 6 hours 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area on each of 6 normal rabbits. The incubation period and the approximate size of the resulting lesions in these inoculated animals are shown in Table II.

In general the incubation period of the syphilitic lesions which developed in the test rabbits was about the same for the 2 "normal" areas in the same animal, but in different animals the incubation period in these areas ranged from 12 to 24 days. It is evident that

rabbits, even from the same stock and of approximately the same age
vary considerably in their reaction to the same inoculum. In the

TABLE II

Results of Protection Tests with Serum from Normal and Syphilitic Rabbits
Experiment 1

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Duration of observation	Interpretation of test Degree of protection
		days		*days*	
1-79	Normal A	12	19	51	Control
	Normal B	12	18	"	"
	46-75	Neg.	0	"	Definite
	46-87	26†	3	"	"
1-80	Normal A	24	17	"	Control
	Normal B	24	19	"	"
	46-75	33	8	"	Definite
	46-87	Neg.	0	"	"
1-81	Normal A	19	24	41	Control
	Normal B	21	24	"	"
	46-75	24†	2	"	Definite
	46-87	Neg.	0	"	"
1-82	Normal A	18	18	"	Control
	Normal B	18	18	"	"
	46-75	28†	3	"	Definite
	46-87	24	6	"	"
1-83	Normal A	23	24	51	Control
	Normal B	22	24	"	"
	46-75	Neg.	0	"	Definite
	46-87	"	0	"	"
1-84	Normal A	24	9	50	Control
	Normal B	24	8	"	"
	46-75	33	4	"	Definite
	46-87	Neg.	0	"	"

* See page 872.
† Pattern of lesions not complete.

12 areas inoculated with immune serum-spirochete mixtures either
no lesions at all developed during the period of observation or, when

lesions did develop, the incubation period was significantly longer than for those in the normal areas. Likewise, the size of such lesions was uniformly smaller than the size of the lesions in the normal areas. In 3 areas inoculated with immune serum mixtures, even though lesions did develop, they did not appear at each inoculated site and the typical pattern was not observed. Photographs of the inoculated areas in rabbits 1-79 and 1-83 are shown in Fig. 1.

It seems evident, therefore, that the serum of these two untreated immune syphilitic rabbits (46-75 and 46-87) possessed some power to inhibit the development of syphilitic lesions in previously uninfected rabbits, when compared under identical experimental conditions with serum from normal rabbits.

Experiment 2.—In this experiment sera from 10 immune syphilitic rabbits were tested for protective power in comparison with two lots of pooled serum from normal rabbits. The duration of infection in the immune rabbits at the time of bleeding is shown in Table IV. Rabbits 47-70, 47-72, and 47-73 had been infected by intracutaneous inoculation and all the others by intratesticular inoculation. No antisyphilitic treatment had been given. Rabbits 46-75, 46-87, 47-70, 47-72, 47-73, and 8-9 were shown to be resistant upon reinoculation of virulent *T. pallidum*. Rabbits 1-3, 1-4, 1-11, and 1-12 were not tested by reinoculation, but it is assumed that they would have shown a chancre immunity. Both normal A and normal B serum consisted of pooled serum from 3 normal rabbits. All animals were bled the day before the test was made, the serum meanwhile being kept in the refrigerator. The inoculum consisted of 1 part of a 25 per cent testicular emulsion, containing numerous *T. pallidum*, to 4 parts of serum. The spirochete emulsion had been frozen prior to inoculation. The serum-spirochete mixtures were incubated at 37°C. for 6 hours. Following incubation, 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area in each of 5 normal rabbits.

The results of these inoculations are shown in Table III. Of the animals inoculated, one (2-19) died prematurely and was omitted from the protocol; another (2-07) failed to develop lesions at the site of the control inoculations and has been classified as unsatisfactory; and in rabbit 2-17 two inoculated areas, obscured by pigmentation and irregular thickening of the skin, were classified as unsatisfactory. Altogether there were 90 satisfactory inoculated areas in 23 animals. Of these areas, 45 were inoculated with one of the normal serum-spirochete mixtures and an equal number with one or another of the

immune serum-spirochete mixtures. Considering all the immune areas as a group, 37 areas inoculated with immune serum mixtures showed definite evidence of the protective power of the immune serum as compared with the normal serum; in 5 areas the evidence of protection was only questionable; and in 3 areas no protective power was manifest.

Again, it is noted that the incubation period of the syphilitic lesions developing in the normal areas was about the same, in most animals, for the two normal serum mixtures, although in one animal (2-02) there was a difference of 5 days. In those animals in which lesions developed at the site of inoculation of immune serum mixtures the incubation period of these lesions was usually significantly longer than in the control areas. As a rule the relative size of the lesions in the different areas showed a direct correlation with the incubation period, the shorter the incubation period, the larger the lesions. Exceptions to this rule are noted, however. Drawings made from representative test animals (2-05, 2-11, 2-16, and 2-21) are shown in Text-fig. 2, and a photograph of the lesions in rabbit 2-21 is shown in Fig. 1.

In the interpretation of the results of these tests, both the incubation period and the size of the lesions are considered. Doubtless, in the case of some areas, all observers might not make the same interpretation of the results in the test animal, but there can be no question that, on the whole, the immune serum exerted an inhibitory effect on the development of syphilitic lesions in those areas when compared with the results in the areas injected with normal serum mixtures. Even in those areas designated as showing questionable protection, either the incubation period of the lesions was slightly longer than that of the controls or else the lesions did not reach the size of the control lesions.

In Table IV is given a summary of the results obtained with the serum from each immune rabbit. Serum from rabbits 47-72 and 47-73 tended to show somewhat less protective power than did serum from the other animals; it may be significant that these animals were originally inoculated intracutaneously. It should be noted, also, that rabbit 1-11, at the time of bleeding had an active syphilitic testicu-

TABLE III

Results of Protection Tests with Serum from Normal and Syphilitic Rabbits

Experiment 2

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection	Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*				*days*		*days*	
2-01	Normal A	20	12	35	Control	2-11	Normal A	20	12	43	Control
	Normal B	22	12	"	"		Normal B	20	12	"	"
	46-75	Neg.	0	"	Definite		47-73	27	4	"	Definite
	46-87	Neg.	0	"	"		1-3	Neg.	0	"	"
2-02	Normal A	16	24	"	Control	2-12	Normal A	22	24	36	Control
	Normal B	21	22	"	"		Normal B	26	16	"	"
	46-75	29†	3	"	Definite		47-73	30	9	"	Definite
	46-87	34	8	"	"		1-3	30	13	"	"
2-03	Normal A	31	12	41	Control	2-13	Normal A	27	9	41	Control
	Normal B	31	9	"	"		Normal B	27	8	"	"
	46-75	34	4	"	Definite		47-73	27	6	"	None
	46-87	34	5	"	"		1-3	27	5	"	Quest.
2-04	Normal A	27	12	"	Control	2-14	Normal A	16	12	36	Control
	Normal B	27	12	"	"		Normal B	16	18	"	"
	46-75	Neg.	0	"	Definite		47-73	15	12	"	None
	46-87	36	6	"	"		1-3	27†	3	"	Definite
2-05	Normal A	21	18	35	Control	2-15	Normal A	23	6	41	Control
	Normal B	18	23	"	"		Normal B	22	12	"	"
	46-75	Neg.	0	"	Definite		47-73	Neg.	0	"	Definite
	46-87	31	5	"	"		1-3	"	0	"	"
2-06	Normal A	29	7	41	Control	2-16	Normal A	10	19	35	Control
	Normal B	27	10	"	"		Normal B	13	20	"	"
	47-70	Neg.	0	"	Definite		1-4	29	4	"	Definite
	47-72	29†	2	"	Quest.		8-9	29	7	"	"
2-07	Normal A	Neg.	0	"	Unsatis.	2-17	Normal A	18	13	41	Control
	Normal B	"	0	"	"		Normal B	Area obscured	0	"	Unsatis.
	47-70	"	0	"	"		1-4	Neg.	0		Definite
	47-72	"	0	"	"		8-9	Area obscured			Unsatis.
2-08	Normal A	13	12	"	None	2-18	Normal A	15	10	37	Control
	Normal B	13	12	"	"		Normal B	13	18	"	"
	47-70	15	9	"	Quest.		1-4	29	3	"	Definite
	47-72	13	12	"	None		8-9	27	6	"	"

* See page 872.

† Pattern of lesions not complete.

TABLE III—*Concluded*

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection	Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*				*days*		*days*	
2-09	Normal A	16	12	43	None	2-20	Normal A	24	5	43	Control
	Normal B	16	8	"	"		Normal B	20	12	"	"
	47-70	27	6	"	Definite		1-4	Neg.	0	"	Definite
	47-72	34	5	"	"		8-9	"	0	"	"
2-10	Normal A	15	18	36	None	2-23	Normal A	15	12	41	Control
	Normal B	16	18	"	"		Normal B	15	10	"	"
	47-70	18	4	"	Definite		1-11	16	6	"	Quest.
	47-72	18	4	"	"		1-12	16†	1	"	Definite
2-21	Normal A	16	24	35	Control	2-24	Normal A	18	17	35	Control
	Normal B	16	24	"	"		Normal B	18	17	"	"
	1-11	27	10	"	Definite		1-11	22	17	"	Quest.
	1-12	34†	5	"	"		1-12	Neg.	0	"	Definite
2-22	Normal A	18	11	41	Control	2-25	Normal A	18	18	37	Control
	Normal B	18	9	"	"		Normal B	18	18	"	"
	1-11	27	9	"	Definite		1-11	27†	3	"	Definite
	1-12	29†	2	"	"		1-12	Neg.	0	"	"

lar lesion. While it seems likely that this animal would have exhibited a chancre immunity on reinoculation at that time, it is probable that its resistance was not as great as that of some of the other animals.

Experiment 3.—In the preceding experiments tests with serum from immune syphilitic animals were controlled with serum from normal rabbits which, as a rule, had been in the laboratory for a much shorter period of time than had the immune animals. Perhaps, too, animals that were the source of the control serum were, on the whole, younger than the immune animals. It is not known whether either of these factors, *i.e.*, duration of time in the laboratory or age of the rabbit, has an appreciable effect on the power of serum to protect against the development of syphilitic lesions, but Experiment 3 was designed to test this point.

Serum was obtained from 2 immune syphilitic rabbits, Nos. 8-9 and 2-54. Both animals had been inoculated intratesticularly, the former 17 months previously and the latter 7 months previously. Typical syphilitic orchitis developed in each, but at the time of bleeding there was no evidence of active syphilitic lesions. No

antisyphilitic treatment had been given. After bleeding, both animals were reinoculated intracutaneously with a homologous strain of *T. pallidum* and both

Days After Inoculation	Inoculated Areas			
	I. Normal A	II. Normal B	III. 46-75	IV. 46-87
15	Negative	Negative	Negative	Negative
21	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative
24	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative
28	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative
33	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative

TEXT-FIG. 2 *a*

Days After Inoculation	Inoculated Areas			
	I. Normal A	II. Normal B	III. 47-73	IV. 1-3
18	Negative	Negative	Negative	Negative
22	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative
25	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative
29	*(lesion drawing)*	*(lesion drawing)*	*(lesion drawing)*	Negative
34	*(lesion drawing)*	*(lesion drawing)*	*(lesion drawing)*	Negative
41	*(lesion drawing)*	*(lesion drawing)*	Negative	Negative

TEXT-FIG. 2 *b*

TEXT-FIG. 2. Schematic drawing of lesions in inoculated areas in 4 rabbits belonging in Experiment 2. The square area of each lesion is reproduced in the drawing. The crossed lines represent degrees of elevation or induration of the lesion. The sera in the serum-spirochete mixtures inoculated into the different areas were as follows:

(*a*) Rabbit 2-05. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 46-75, immune. IV, rabbit 46-87, immune.

(*b*) Rabbit 2-11. I, normal A (3 pooled). II, normal B (3 pooled). III rabbit 47-73, immune. IV, rabbit 1-3, immune.

remained negative for a period of 60 days. Controls similarly inoculated developed typical syphilitic lesions within 30 days.

TEXT-FIG. 2c

TEXT-FIG. 2d

(c) Rabbit 2-16. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-4, immune. IV, rabbit 8-9, immune.

(d) Rabbit 2-21. I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-11, immune. IV, rabbit 1-12, immune.

While in the 4 rabbits represented here, the two normal serum-spirochete mixtures were inoculated in areas I and II, respectively, in other rabbits of this experiment these mixtures were inoculated in different areas.

Source of "immune" serum tested			Results of test in inoculated rabbits			
				Number showing		
Rabbit No.	Site of original inoculation	Duration of infection	Number inoculated	Definite protection	Questionable protection	No protection
		mos.				
46-75	Testis	24	5	5	0	0
46-87	"	22	5	5	0	0
47-70	Skin	17	4	3	1	0
47-72	"	17	4	2	1	1
47-73	"	17	5	3	0	2
1-3	Testis	10	5	4	1	0
1-4	"	10	4	4	0	0
8-9	"	10	3	3	0	0
1-11	"	6*	5	3	2	0
1-12	"	6	5	5	0	0

* Active testicular lesions at time of bleeding.

TABLE V

Results of Protection Tests with Serum from Immune Syphilitic Rabbits and with Serum from Normal Rabbits Which Had Been Maintained under Laboratory Conditions over a Period of Months

Experiment 3

Test rabbit No.	Source of serum Rabbit No.	Incubation period	Size of lesions*	Observed	Interpretation of test Degree of protection
		days		*days*	
4-02	1-94 (normal)	30	8	34	Control
	3-13 (normal)	30	10	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-03	1-94	18	10	22	Control
	3-13	18	11	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-04	1-94	21	6	29	Control
	3-13	20	18	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"
4-05	1-94	23	7	"	Control
	3-13	19	16	"	"
	8-9	Neg.	0	"	Definite
	2-54	"	0	"	"

* See page 872.

As controls in this experiment serum was obtained from 2 normal rabbits, Nos. 1-94 and 3-13. The former animal had been in the laboratory 8 months at the time of bleeding and the latter animal 7 months. One part of a 50 per cent testicular emulsion, containing numerous active *T. pallidum*, was added to 9 parts of serum. The mixture was incubated at 37°C. for 6 hours and at the end of this period 0.1 cc. of each mixture was inoculated intracutaneously in 6 sites of one area in each of 4 normal rabbits.

The results of these inoculations are shown in Table V. In each of the test rabbits a typical pattern of syphilitic lesions developed in the areas inoculated with normal serum-spirochete mixtures, while not a single lesion developed during the period of observation in any area inoculated with immune serum-spirochete mixtures. (See photograph of rabbit 4-03, Fig. 1.) The conclusion must be drawn that the serum from the immune animals inhibited the development of syphilitic lesions under the conditions of this experiment. While the results are not conclusive, the experiment indicates that simply maintaining normal rabbits under laboratory conditions for a period of months does not serve appreciably to increase the titer of protective antibodies against *T. pallidum*.

DISCUSSION

Most writers reviewing the large amount of experimental work of the past 30 years on the nature of immunity in syphilis have concluded, and rightly so, on the basis of the available evidence, that this acquired resistance is a property primarily of the fixed tissue cells. Despite the fact that both man and animals, under certain conditions, exhibit a high degree of resistance to reinoculation, only an occasional investigator has been able to obtain evidence of a humoral expression of this immunity. In reviewing the experiments bearing directly on this point, however, it is evident that in many instances the methods of testing for the presence of humoral antibodies specific for *T. pallidum* were relatively crude compared with the techniques now available.. Much more is now known concerning the general course of experimental syphilis and, in particular, much more is known of the various factors that influence the development of the immune state.

In the experiments reported in this paper liberal use has been made of techniques developed in the study of filtrable viruses and other

infectious agents. Likewise, improved technical methods in experimental syphilis have made it possible to control certain variable elements which materially affect the reaction between host and parasite. For example, a large amount of infectious material can be prepared at once, its relative infectivity for rabbits can be determined, and the whole lot of material can be preserved essentially unaltered over long periods of time by freezing at low temperatures. Thus, it is practicable fairly regularly to employ in the protection test an inoculum which closely approaches the minimal chancre dose of *T. pallidum*. Perhaps this feature is the most important element in the protection tests described above. It is probable that the inocula ordinarily used in experimental syphilis vary widely in their degree of infectivity for normal animals, which may in large measure explain the negative or equivocal results obtained by other investigators in their attempt to demonstrate protective antibodies in syphilis.

From the foregoing experiments it seems clear that the serum of rabbits which had had syphilis for 6 months or longer and had not been treated exerted a treponemicidal or treponemistatic effect on virulent *T. pallidum* belonging to a homologous strain, as compared with the effect of serum from non-syphilitic rabbits. It is not known whether this effect was exerted *in vitro* or only after injection of the mixtures into a living host. Nor is the mechanism of this action known. Because prolongation of the incubation period of the serum-spirochete mixtures seemed to accentuate the differences between normal and immune serum, it is assumed that some change occurs *in vitro*. Other evidence is also available which suggests that this may be a direct treponemicidal effect. Since, however, under conditions of these experiments spirochetes are present in the mixtures in only relatively small numbers, the point is difficult to determine. Experiments bearing on this question are in progress.

It seems likely that active complement must be present in serum-spirochete mixtures in order to demonstrate this protective action. In experiments in which the complement in the serum was inactivated, much poorer and much more variable results were obtained than when unheated serum was tested. It is probable, however, that some complement is usually present in the testicular tissue emulsion and

this amount may be sufficient in many instances to fulfill the requirements of the reaction.

In these experiments no effort has been made to determine the titer of the protective antibodies. Probably an excess of serum was used in the test. It may be possible to reduce the amount of serum to the point where smaller differences in antibody content can be detected.

These experiments and those of several other workers mentioned above indicate that specific humoral antibodies are produced during the course of syphilitic infection in rabbits. If this is true, it would serve to remove syphilis from the rather unique position among infectious diseases which it has occupied in the past, and place it among the ever growing group of diseases in which resistance to reinfection is associated at some period during the course of the infection with the presence of humoral antibodies specific for the causative agent of that disease. This phenomenon is observed more characteristically in the acute infections. The mere fact that a disease is chronic and the infection ordinarily of long duration in itself suggests that the resistance to the causative organisms, whether to those already within the body, or to those that reach it from without, is not highly developed. By the same reasoning, it could be hypothesized that the humoral expression of this immunity would, likewise, probably be only imperfectly developed. This seems to be the case in syphilis.

SUMMARY

1. When an emulsion containing virulent *Treponema pallidum* is added to serum from normal rabbits and from untreated immune syphilitic rabbits that have been infected with a homologous strain of *T. pallidum*, the mixture incubated at 37°C., and injected intracutaneously into normal rabbits, typical syphilitic lesions commonly develop at the sites of inoculation of the normal serum-spirochete mixture, while at the sites of inoculation of immune serum-spirochete mixtures usually either no lesion develops or else the incubation period of the resulting lesions is shorter and the lesions remain smaller than those produced by normal serum-spirochete mixtures.

2. In a series of preliminary experiments, of 56 areas inoculated with serum-spirochete mixtures, in 42 the suppressive action of the

syphilitic serum was manifest, in 10 areas questionable evidence of protection was noted, and in 4 areas there was no evidence that the syphilitic serum had exerted a suppressive or protective action.

3. The protective action of syphilitic serum seems to have been lessened by heating to 56°C.

4. The results of the protection test in three other series of experiments were as follows: (*a*) Of 12 areas in 6 rabbits inoculated with normal serum-spirochete mixtures typical syphilitic lesions developed, while in the same number of areas inoculated with immune serum-spirochete mixtures there was complete or partial suppression of lesions in all. (*b*) Of 45 areas inoculated with serum from 10 different immune syphilitic rabbits, definite evidence of protection was observed in 37, questionable evidence in 5, and no evidence of protection in 3. (*c*) Of 8 areas in 4 rabbits inoculated with immune serum-spirochete mixtures no lesions developed during the period of observation, while of 8 areas in the same rabbits inoculated with one of two normal serum-spirochete mixtures typical syphilitic lesions developed in each.

CONCLUSION

During the course of syphilitic infection rabbits develop specific humoral antibodies which can be demonstrated by an appropriate "protection test." The presence of these antibodies is associated with a high degree of acquired immunity to the disease.

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EXPLANATION OF PLATE 44

FIG. 1. Photographs of the backs of rabbits inoculated with serum-spirochete mixtures.

(a) Rabbit 1-79. Experiment 1. Excised skin of back, 51 days after inoculation. Areas inoculated with the following sera: I, rabbit 46-75, immune. II, normal A (2 pooled). III, rabbit 46-87, immune. IV, normal B (2 pooled). Note pattern of large syphilitic lesions in areas II and IV. There are no lesions in areas I and III, although these areas are partially obscured by pigment.

(b) Rabbit 1-83. Experiment 1. Excised skin of back, 51 days after inoculation. Areas inoculated with the following sera: I, rabbit 46-75, immune. II, normal A (2 pooled). III, rabbit 46-87, immune. IV, normal B (2 pooled). Note pattern of large syphilitic lesions in areas II and IV and absence of lesions in areas I and III.

(c) Rabbit 2-21. Experiment 2. Excised skin of back, 35 days after inoculation. Areas inoculated with the following sera: I, normal A (3 pooled). II, normal B (3 pooled). III, rabbit 1-11, immune. IV, rabbit 1-12, immune. Note pattern of syphilitic lesions in areas I and II, and absence of lesions in areas III and IV.

(d) Rabbit 4-03. Experiment 3. Excised skin of back 22 days after inoculation. Areas inoculated with the following sera: I, rabbit 1-94, normal. II, rabbit 2-54, immune. III, rabbit 3-13, normal. IV, rabbit 8-9, immune. Note pattern of small lesions in areas I and III, and absence of lesions in areas II and IV.

Fig. 1

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